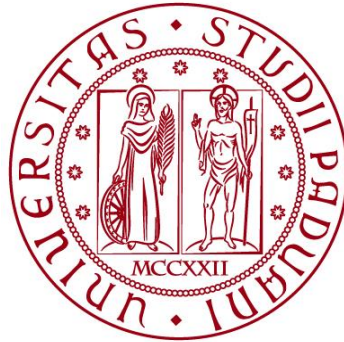


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biologia Molecolare



ELABORATO DI LAUREA

**Coinvolgimento della N-glicosilazione dei residui
Asn331 e Asn343 della proteina spike di SARS-CoV-2
nel legame con ACE2, nell'ingresso del virus e nella
regolazione di IL-6**

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Abstract

SARS-CoV-2 è l'agente eziologico della pandemia di COVID-19 che ha provocato una crisi della salute pubblica globale. SARS-CoV-2 penetra all'interno delle cellule bersaglio attraverso un'interazione molecolare tra la glicoproteina virale spike (S) ed il recettore presente sulla superficie cellulare, la proteina ACE2. È stato dimostrato che la glicosilazione delle proteine virali svolge un ruolo importante nell'infettività. Lo studio di Das e Collaboratori riporta che la N-glicosilazione dei residui N331 e N343 del Receptor Binding Domain (RBD) della spike i) è richiesta per il legame ad ACE2, interagendo con uno specifico epitopo glucidico caratterizzato da un galattosio terminale (G4G), ii) influenza le prime fasi del ciclo replicativo virale ed iii) interferisce con la regolazione trascrizionale della molecola di segnale pro-infiammatoria IL-6. Questi risultati indicano che la glicosilazione dei residui N331 e N343 di RBD è cruciale nella interazione virus-cellula ospite e nel processo di patogenesi dell'infezione da SARS-CoV-2.

Key words: ACE2, COVID-19, N-glicosilazione, proteina spike, SARS-CoV-2

1. Introduzione

La pandemia di COVID-19 (Coronavirus Disease 19) permane un problema globale di salute pubblica, con oltre 676 milioni di casi riportati e 6 milioni di vittime (dati riferiti a maggio 2023). Numerosi approcci terapeutici sono stati testati per il trattamento della COVID-19 (es. Remdesivir), ma in numero limitato hanno dimostrato un'efficacia effettiva nei trial clinici. Inoltre, le cure ospedaliere per i pazienti e il trattamento delle complicazioni come la tempesta citochinica e l'insufficienza degli organi nei casi severi aumentano l'impatto sulle strutture sanitarie.

1.1 SARS-CoV-2

L'agente eziologico della COVID-19 è il SARS-CoV-2 (Severe Acute Respiratory Sindrome Coronavirus 2). SARS-CoV-2 appartiene all'ordine *Nidovirales*, famiglia *Coronaviridae*, sottofamiglia *Orthocoronavirinae*, genere *Betacoronavirinae* e sottogenere *Sabercovirus*. I *Coronavirus* sono un gruppo eterogeneo di virus sferici provvisti di envelope, con un capsido a simmetria elicoidale ed un genoma a RNA a singolo filamento a polarità positiva (ssRNA+). Possiedono il genoma di più grandi dimensioni (26,4-31,7kbp) tra tutti i virus a RNA, con un *GC content* che varia tra il 32 e il 43%. Il genoma di SARS-CoV-2 è caratterizzato da 6 *open reading frames* (ORFs) funzionali e 7 ORFs codificanti proteine accessorie, intersperse tra i geni strutturali. In dettaglio il genoma codifica 4 proteine strutturali, 16 proteine non strutturali (Nps) e molte proteine accessorie. Le proteine strutturali sono:

- N: proteina del nucleocapside (419 aa), coinvolta nel packaging del genoma virale, nell'assemblaggio, nella gemmazione e nella formazione completa del virus;
- M: proteina di membrana (222 aa), principale proteina della matrice virale, interagisce con le altre proteine strutturali nelle fasi di assemblaggio e rilascio di nuove particelle virali;
- E: proteina dell'envelope (75 aa), la più piccola delle proteine strutturali, partecipa all'assemblaggio ed alla gemmazione virale (l'assenza di tale proteina può determinare un blocco nella maturazione di nuovi virioni o la generazione di progenie virale incompetente nella replicazione);
- S: proteina spike di superficie (1273 aa e 150 kDa), appartenente alla classe I delle glicoproteine transmembrana. Le proteine S sono organizzate in modo radiale sulla superficie del virus, conferendo ad esso un caratteristico aspetto a corona solare. La proteina S è formata da due subunità maggiori: S1 e S2. La subunità N-terminale S1 possiede due domini strutturali ben definiti: il dominio di legame al recettore (RBD) ed il dominio *galectin-like* N-terminale (S-NTD); partecipa al riconoscimento ed al legame del recettore cellulare. La subunità C-terminale S2 contiene cinque domini funzionali: un peptide di fusione (FP), le regioni *heptad repeat* N- e C-terminale (HR-N e HR-C), un dominio transmembrana (TM) ed un dominio citoplasmatico (CT). S2 facilita il processo di fusione virale con la

membrana cellulare attraverso l'interazione con la serina proteasi transmembrana 2 (TMPRSS2) presente sulla superficie della cellula.

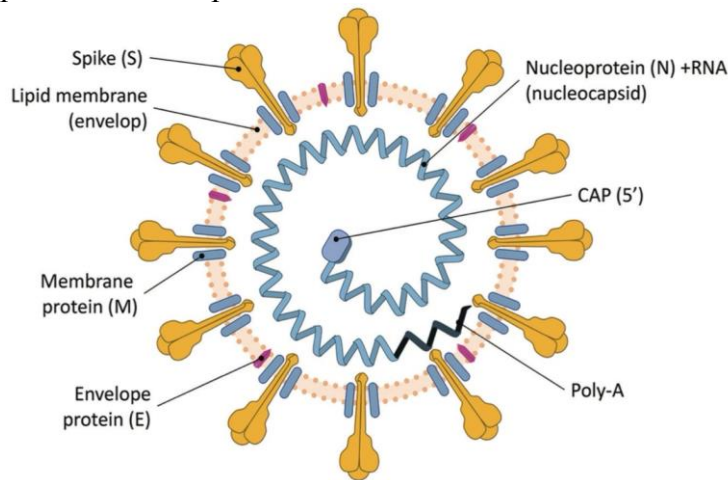


Figura 1: Diagramma semplificato di SARS-CoV-2^[1]

Il ciclo replicativo di SARS-CoV-2 include cinque fasi: adsorbimento, penetrazione, biosintesi, maturazione e rilascio.

La fase iniziale prevede l'attacco di RBD al recettore ACE2 della cellula ospite. RBD è una regione di 211 amminoacidi della subunità S1 della proteina spike, avente un ruolo chiave nella fase di ingresso del virus; è formato da due domini strutturali: il core e il dominio esterno. Il core è altamente conservato ed è composto da 5 foglietti beta disposti in modo antiparallelo e un ponte disolfuro che li lega. Il dominio esterno presenta un loop, stabilizzato da un ponte disolfuro. RBD passa continuamente da una posizione aperta (*pre-fusing upright*), che lega il recettore, ad una posizione chiusa (*post-fusing down*), importante nella neutralizzazione da parte degli anticorpi. Nel contempo, la subunità S2 recluta l'enzima TMPRSS2 che catalizza il taglio della spike a livello del sito di clivaggio S2', in un processo noto come *priming*. Il priming libera la proteina spike per la fusione con la membrana della cellula ospite e successivamente per l'ingresso nella cellula. Il legame del recettore destabilizza la struttura di pre-fusione portando alla dissociazione della subunità S1 e al refolding della subunità S2 in una conformazione stabile di post-fusione. L'envelope si fonde poi con gli endosomi di membrana e rilascia il nucleocapside virale nel citoplasma. L'RNA genomico è qui tradotto dai ribosomi cellulari per generare una RNA polimerasi RNA dipendente, che catalizza successivamente la sintesi di un RNA- *full strand* complementare al genoma. La trascrizione discontinua che utilizza tale RNA- come stampo permette di generare un set di 5-7 mRNA subgenomici e l'RNA genomico *full-length*. Sequenze uniche presenti all'estremità 5' degli mRNA sono tradotte per produrre le proteine strutturali e non strutturali. Le proteine strutturali si assemblano per formare il nucleocapside a simmetria elicoidale, includendo l'RNA genomico di nuova sintesi. I nuovi virioni si formano gemmando nel lume dei compartimenti intermedi del Reticolo Endoplasmatico-Golgi (ERGIC); tali vescicole fondono poi con la membrana plasmatica e vengono rilasciati dalla cellula infettata per esocitosi.

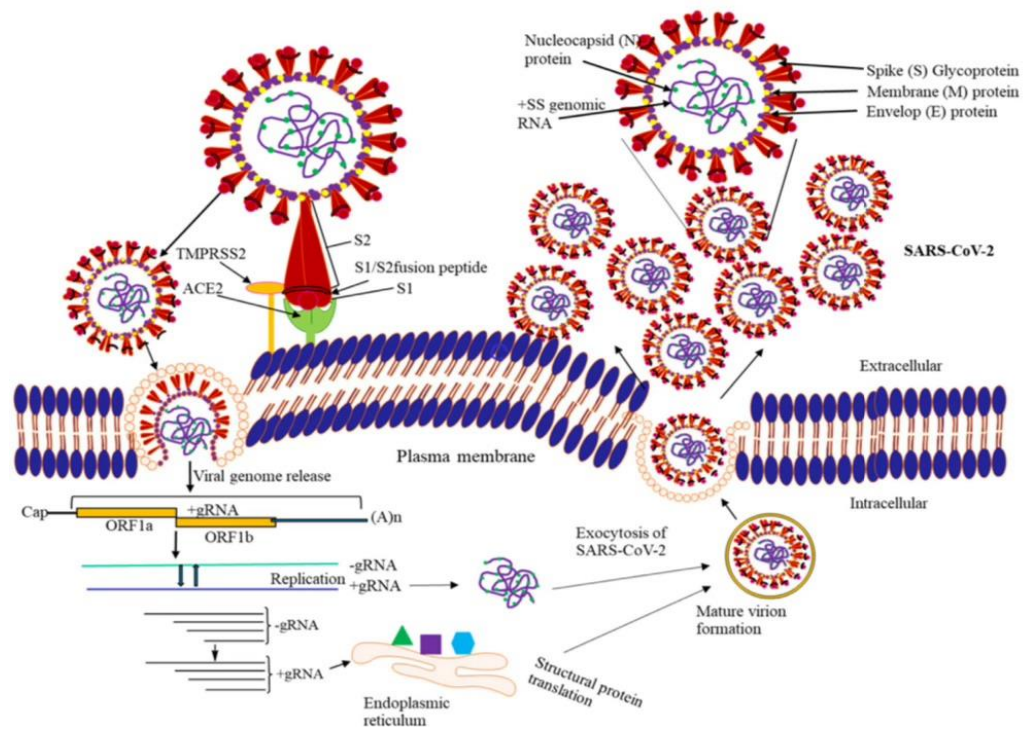


Figura 2: Ciclo replicativo di SARS-CoV-2^[3]

Una caratteristica importante della proteina spike di SARS-CoV-2 è l'estensiva glicosilazione.

1.2 Ruolo della glicosilazione nella patogenesi virale

I virus rivestiti sono caratterizzati dalla presenza di un envelope: un *bilayer* lipidico di derivazione cellulare, modificato per inserzione di proteine virali glicosilate dal macchinario di glicosilazione della cellula. La glicosilazione prevede il legame di zuccheri alle proteine ed è una delle modifiche post-traduzionali più importanti. Si distinguono N- ed O-glicosilazione. La N-glicosilazione comporta il legame covalente di un core ad alto contenuto di mannosio al gruppo amminico di un residuo di asparagina, facente parte della sequenza Asn-X-Ser/Thr (X≠Pro). Tale legame avviene precocemente nel corso della sintesi della proteina ed è seguito da un processo complesso di *trimming* e di rimodellamento dell'oligosaccaride legato durante il transito attraverso reticolo endoplasmatico e Golgi.

Nei virus, la N-glicosilazione ha implicazioni importanti per quanto concerne: i) ciclo virale, in particolare nelle fasi di adsorbimento e penetrazione (per interazione con il recettore cellulare) e di uscita (nel corso del processo di gemmazione); ii) folding, struttura, trasporto e stabilità delle proteine virali; iii) antigenicità, infettività e virulenza: i glicani possono nascondere un epitopo antigenico, che consente al virus di evitare il riconoscimento da parte degli anticorpi neutralizzanti, fornendo una via conveniente per infettare nuove cellule ospiti.

La proteina S di SARS-CoV-2 è estensivamente glicosilata: 22 siti di N-glicosilazione e molti siti di O-glicosilazione.

Tipi specifici di glicani sono stati osservati a livello di ogni sito della proteina S e la glicosilazione nella regione RBD è critica per l'infettività virale.

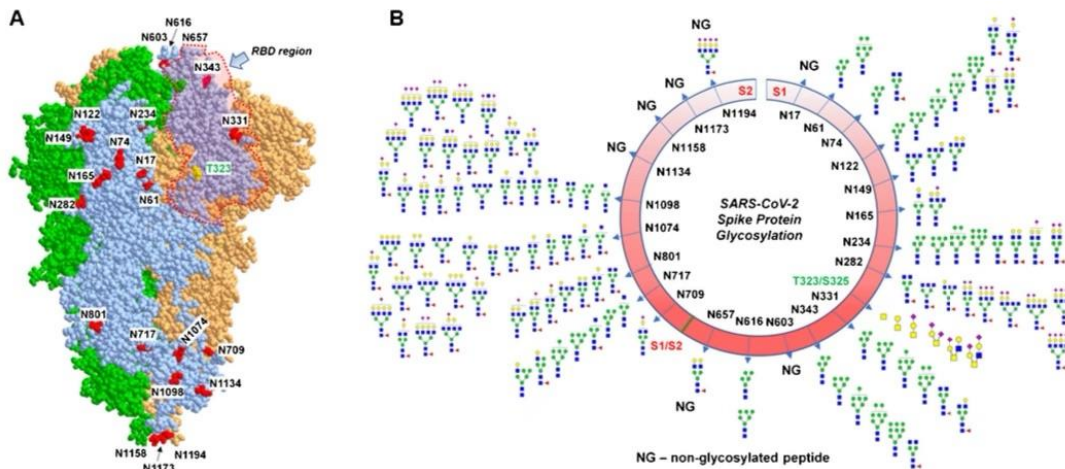


Figura 3: Glicosilazione della proteina spike (S) di SARS-CoV-2. **A.** Modello 3D della proteina S che mostra la regione RBD e i siti di glicosilazione. **B.** Siti specifici di N- e O-glicosilazione della proteina spike^[5]

L'articolo riporta che la N-glicosilazione di SARS-CoV-2 è essenziale per il binding del recettore ACE2 e per l'ingresso del virus nella cellula ospite. In particolare, conferma che i residui Asn331 e Asn343 di RBD giocano un ruolo chiave nel legame al recettore cellulare e nell'infettività virale. Viene dimostrato, inoltre, che c'è una up-regolazione dell'espressione di IL-6 nel corso dell'infezione da SARS-CoV-2 e che la N-glicosilazione dei residui N331 e N343 è coinvolta nella sua regolazione. Infine, viene riportato che il glicano Gal- β -1,4-GlcNAc rappresenta l'epitopo di legame dell'RBD.

2. Approccio sperimentale: metodi

2.1 Spike/ACE2 binding assay

Per analizzare l'interazione tra il receptor binding domain (RBD) della proteina spike ed il recettore cellulare angiotensin-converting enzyme 2 (ACE2) è stato usato il metodo ELISA (enzyme-linked immunosorbent assay). In una micropiastra da 96 pozzetti, rivestita con RBD ricombinante, è stata aggiunta la proteina ACE2 umana in diverse concentrazioni. La piastra è stata quindi incubata con anticorpi anti-ACE2 di capra. Dopo l'aggiunta di IgG anti-capra coniugate con HRP (Horseradish peroxidase), i segnali sono stati generati con il substrato 3,3',5,5'-tetrametilbenzidina e letti da un colorimetro a lunghezza d'onda di 450nm.

L'interazione RBD/ACE2 è stata valutata anche deglicosilando l'una o l'altra proteina o entrambe. La deglicosilazione è stata eseguita in condizioni native o ridotte usando Protein Deglycosylation Mix II (New England Biolabs, cat. P6044) oppure deglicosilasi individuali (PNGase F, O-glycosidase, α 2-3,6,8,9 neuraminidase A, β 1-4 galactosidase S, e β -N-acetylhexosaminidasef (New England Biolabs)). Sono seguiti un'elettroforesi SDS-PAGE e un immunoblot (gli

anticorpi di topo anti-proteina S di SARS-CoV-2 sono stati usati alla concentrazione di 1µg/mL).

Il binding assay è stato usato anche per determinare cambiamenti nel legame (calcolati in %) tra RBD e il recettore ACE2 sottoposto ad un trattamento con neuroamminidasi. La proteina ACE2 trattata con la neuroamminidasi è stata incubata per 1 ora a 37°C, incluso il controllo (senza enzima).

Protein	Species	GenBank	Expressed region	Expression system	Source
S1 subunit, RBD	SARS-CoV-2	QHD43416	Arg319-Phe541	HEK293	RayBiotech (cat. 230-30162)
S1 subunit, RBD	SARS-CoV-2	QHD43416	Arg319-Phe541	<i>Escherichia coli</i>	RayBiotech (cat. 230-01102)
S2 subunit	SARS-CoV-2	QHD43416	Met697-Pro1213	HEK293	RayBiotech (cat. 230-30163)
Nucleocapsid	SARS-CoV-2	QHD43423	Met1-Ala419	HEK293	RayBiotech (cat. 230-30164)
ACE2	Human	Q9BYF1	Gln18-Ser740	HEK293	RayBiotech (cat. 230-30165)
p24	HIV	Q9WMW5	Ser5-Cys207	<i>E. coli</i>	RayBiotech (cat. 230-00505)
S1 subunit, RBD, L452R/T478K mutant	SARS-CoV-2	QHD43416	Arg319-Phe541	HEK293	RayBiotech (cat. 230-30208)
Spike RBD, B.1.1.529	SARS-CoV-2	YP_009724390.1	Arg319-Phe552	HEK293	SinoBiological (cat. 40592-V08H121)

Tabella 1: Proteine ricombinanti usate nel binding assay

2.2 Colture cellulari

Sono state usate due linee cellulari, fornite dall'ATCC (American Type Culture Collection), con un'alta espressione di ACE2: Vero e A549. Le cellule Vero C1008 sono cellule epiteliali di rene di scimmia africana, mentre le cellule A549 sono cellule epiteliali basali di adenocarcinoma umano. Tali cellule sono state coltivate in DMEM (Dulbecco's Modified Eagle Medium) contenente FBS (fetal bovine serum) al 10% e penicillina/streptomycin 1X, a una temperatura di 37°C e 5% di CO₂. Queste due linee cellulari sono state usate per i saggi di titolazione e neutralizzazione con pseudovirus.

Per la produzione di pseudovirus, invece, sono state usate le cellule BHK21/WI-2 che sono cellule di rene di criceto. Queste sono state coltivate in DMEM, FBS al 5%, penicillina/streptomycin, temperatura di 37°C e 5% di CO₂.

2.3 Generazione e titolazione di pseudovirus

All'interno del plasmide pCAGGS-G-kan è stata clonata la sequenza del cDNA codificante la glicoproteina spike wild type o mutante; sono stati creati tre costrutti mutanti di S, usando come vettore template wt/pCAGGS e poi è stato eseguito un sequenziamento per la conferma della avvenuta mutagenesi.

Un giorno prima della trasfezione le cellule BHK21 sono state seminate ad una densità di 3*10⁶ cellule e incubate overnight a 37°C in 5% di CO₂ fino a raggiungere la confluenza di 70-90%. Usando Lipofectamine 3000 Transfection Reagent (Invitrogen), le cellule BHK21 sono state trasfettate con 16µg di plasmide pCAGGS-G-kan. Le cellule trasfettate sono state poi infettate con VSV G*ΔG-luciferasi ad una molteplicità di infezione (MOI) pari a 0,1 e incubate a 37°C in 5% di CO₂ per 1,5 ore, aggiungendo poi DMEM fresco. Dopo 24 ore dall'infezione, il surnatante delle colture contenente VSV G*ΔG-luciferasi è stato raccolto e le aliquote sono state conservate a -80°C fino all'uso.

Per generare pseudovirus di SARS-CoV-2, le cellule BHK21 sono state trasfettate con 16µg di plasmide pCAGGS-SARS-CoV-2 spike. Le cellule trasfettate sono state poi infettate con VSV G*ΔG-luciferasi a MOI di 3-5.

Il titolo degli pseudovirus è stato determinato in cellule Vero, A549, BHK21. Le cellule sono state seminate in piastre da 96 pozzetti con fondo chiaro e sono state infettate con 100µL di pseudovirus diluito in serie in base 2 (1:2) in siero DMEM free (la diluizione iniziale era di 10 volte). La prima linea della piastra è stata trattata come controllo, senza lo pseudovirus (mock infection). Dopo 1,5 ore di incubazione a 37°C in 5% di CO₂, sono stati aggiunti 100µL di DMEM fresco, contenente FBS al 10% e penicillina/streptomina. Dopo 24 ore dall'infezione sono state determinate le unità di luce relativa (RLU) usando il sistema ONE-Glo EX Luciferase Assay e un luminometro.

2.4 Glycan array

Un chip di glycan array è un pannello di 300 glicani, usato come screening high throughput per determinare l'interazione tra RBD e i carboidrati nell'array.

400µL di RBD e proteine mutanti (50ng/mL) sono stati incubati nei pozzetti per 2-3 ore. Dopo lavaggio, sono stati aggiunti anticorpi primari biotinilati (20ng/mL) e il tutto è stato incubato per 1 ora. Dopo lavaggio, sono stati aggiunti anticorpi secondari di topo anti-IgG alla diluizione 1:5000 e l'array è stato incubato per 1 ora. Dopo lavaggio, è stata aggiunta streptavidina coniugata con il colorante equivalente Cy3 e lasciata in agitazione per 1 ora. Infine, il chip è stato lavato con 30mL di acqua distillata o de-ionizzata per 5 minuti. I segnali sono stati visualizzati con lo scanner laser Axon GenePix. L'analisi e la quantificazione dell'immagine è stata fatta usando il software di analisi del microarray.

2.5 Antibody array

L'array L-1000 è un array semi-quantitativo label-based per il rilevamento di mille proteine umane, in cui ogni anticorpo proteina-specifico è stato fissato su una superficie di vetro mentre i campioni sono biotinilati. 120µL di terreno di coltura sono stati purificati con una desalting spin column e poi biotinilati a temperatura ambiente per 30 min. La miscela di reazione è stata poi fatta passare attraverso un'altra colonna per rimuovere la biotina libera. Il campione è stato diluito 10 volte in soluzione Blocking 1X e, dopo i passaggi precedenti, i vetrini sono stati visualizzati usando lo scanner Innopsys 710AL. I dati, estratti con il software Mapix, sono stati analizzati con Excel.

Il Kiloplex antibody array è un array quantitativo sandwich-based per il rilevamento di mille proteine umane. A partire dallo standard Mix sono state allestite delle diluizioni 1:2; l'array è stato incubato a temperatura ambiente con un mix di anticorpi biotinilati e poi lavato, quindi è stato incubato con streptavidina combinata al colorante Cy3. I vetrini sono stati visualizzati in XDR mode usando lo scanner Innopsys 710AL e i dati estratti usando il software Mapix.

Il kit RayBio® Human IL-6 ELISA è un'analisi ELISA in vitro per la misurazione quantitativa della proteina IL-6 umana, utile per la determinazione del livello di IL-6 nei terreni condizionati dallo pseudovirus assay.

2.6 Real-time PCR

Per misurare l'espressione dell'mRNA di IL-6, attraverso il kit RNAeasy Mini (QIAGEN) è stato isolato l'RNA totale dalle cellule HEK-293T e HUVEC trattate con WT-RBD, proteine mutanti, IL-6 umano ricombinante. La sintesi del cDNA è stata fatta usando il kit iScript cDNA synthesis (Bio-Rad). La qPCR è stata allestita sub-iniettando 500ng di cDNA usando il power TRACK Sybr mix e primers specifici per la IL-6 umana (*forward*: 5'-TCGGTCCAGTTGCCTTCT-3'; *reverse*: 5'-TGAGATGCCGTCGAGGAT-3') e per il 18srRNA (*forward*: 5'-TTGGTGGAGCGATTTGTCTG-3'; *reverse*: 5'-ATCTCGGGTGGCTGAACG-3'). I livelli di espressione dell'mRNA di IL-6 sono stati normalizzati con i livelli di 18srRNA. L'espressione relativa dell'mRNA di IL-6 nel controllo non trattato e nei campioni trattati è espressa come valori di $2^{-\Delta Ct}$ e i *fold changes* sono stati calcolati comparando i valori di $2^{-\Delta Ct}$ del campione trattato con quelli del controllo non trattato.

2.7 Lactate dehydrogenase (LDH) assay

Attraverso il kit LDH Cytotoxicity Assay è stata rilevata la quantità di LDH rilasciata, come misura della citotossicità del virus. Cellule HEK-293, A549 e HUVEC sono state seminate in piastre da 12 pozzetti e mantenute overnight prima del trattamento con WT-RBD, proteine mutanti e IL-6 umano ricombinante ad una concentrazione di 1000ng/mL. Dopo 24 ore, il co delle colture è stato raccolto con centrifugazione e testato in triplicato (secondo le linee guida). L'assorbanza (A) è stata misurata a lunghezze d'onda di 490 e 680nm usando uno spettrofotometro e i valori di A sono stati normalizzati per la quantità di proteina nei lisati attraverso quantificazione del BCA (acido bicinconinico). *Fold changes* nella citotossicità sono stati calcolati basandosi sulle differenze rispetto al controllo LDH-positivo fornito dal kit.

2.8 Quantificazione di IL-6 e RBD nel siero di pazienti COVID-19

I campioni di sangue sono stati raccolti da pazienti adulti selezionati basandosi sulla diagnosi di COVID-19 fatta con test RT-PCR approvato dalla FDA (Food and Drug Administration). Il siero è stato usato per la misurazione di IL-6 e RBD. Il sangue intero è stato raccolto in tubi contenenti un gel di separazione e, dopo la centrifugazione, è stato conservato a -80°C. IL-6 e RBD sono stati quantificati usando un kit ELISA.

2.9 Analisi statistica

I dati sono stati espressi come media +/- SEM (Standard Error of the Mean) e sono stati ottenuti da un numero appropriato di esperimenti indipendenti condotti in

duplicato o triplicato. La significatività statistica è stata valutata utilizzando il software GraphPad Prism. Test t a due code non accoppiati o accoppiati hanno permesso di analizzare i dati che coinvolgono un confronto diretto di un gruppo sperimentale con un gruppo di controllo. La ANOVA one- o two-way è stata adoperata con misure ripetute per gruppi sperimentali appropriati e seguita dalla correzione di confronto multiplo di Tukey e Bonferroni. Per gli esperimenti quantitativi di PCR e rilascio di LDH è stato eseguito una ANOVA one-way in modo da determinare il significato dell'espressione del gene target nei gruppi di controllo rispetto a quelli di trattamento. I valori p riportati sono stati adeguati a tenere conto di confronti multipli. Per tutti i test statistici, è stato accettato come statisticamente significativo un livello di confidenza bilaterale di $p < 0,05$ (intervallo di confidenza del 95%).

3. Risultati e discussione

3.1 Coinvolgimento della glicosilazione N-linked della proteina spike nel legame con ACE2 e nell'ingresso intracellulare di pseudovirus

Per determinare il ruolo specifico della glicosilazione nell'interazione e nel legame tra RBD e ACE2, Das et al hanno sviluppato un metodo high throughput di screening in vitro. Piastre da microtitolazione sono state rivestite da diverse molecole: la proteina RBD ricombinante, la subunità S2 della proteina spike, la proteina del nucleocapside N, la proteina p24 di HIV, come controlli negativi. Per testare se l'affinità di legame RBD/ACE2 era influenzata dalle mutazioni associate con le varianti omicron e delta di SARS-CoV-2, il binding assay è stato fatto con RBD delle varianti omicron e delta. A seguito dell'incubazione delle piastre con la proteina ACE2 umana, i ricercatori hanno confermato che ACE2 lega specificatamente RBD e non tutte le altre proteine testate (figura 4a). È stato inoltre dimostrato che la proteina RBD ricombinante generata per over-espressione all'interno di cellule umane HEK293 lega ACE2 con un'affinità significativamente più alta se comparata con RBD ricombinante generato in *Escherichia coli* (figura 4a) in modo dose-dipendente (figura 4b). Questo significa che le modificazioni post-traduzionali specifiche che avvengono negli eucarioti influenzano l'affinità di legame ACE2/RBD. Per quanto riguarda le varianti virali, è stata riscontrata una riduzione modesta dell'affinità solo con la variante omicron (dati non riportati).

Per determinare l'estensione dell'influenza della glicosilazione sull'affinità di legame ACE2/RBD, RBD, generato all'interno di cellule HEK293, è stato deglicosilato per rimuovere i glicani sia N- che O-linked, con conseguente shift della dimensione delle proteine (figura 4c). Nell'articolo viene riportato che RBD lega ACE2 in modo dose dipendente, inoltre la deglicosilazione di RBD abolisce il suo legame (figura 4d). Al contrario, la deglicosilazione di ACE2 ha un impatto minimo sull'interazione; questa evidenza ha suggerito che è la glicosilazione di RBD, e non del recettore, ad essere critica per l'interazione proteina-proteina. In dettaglio, Das et al hanno trattato RBD con diverse deglicosilasi (PNGase F, O-

glycosidase, α 2-3,6,8,9 neuraminidase A, β 1-4-galattosidase S, e β -N-acetylhexosaminidase f). Di tutti gli enzimi testati, solo il trattamento con PNGasi, che taglia gli oligosaccaridi N-linked, diminuisce significativamente l'affinità del legame (figura 4e).

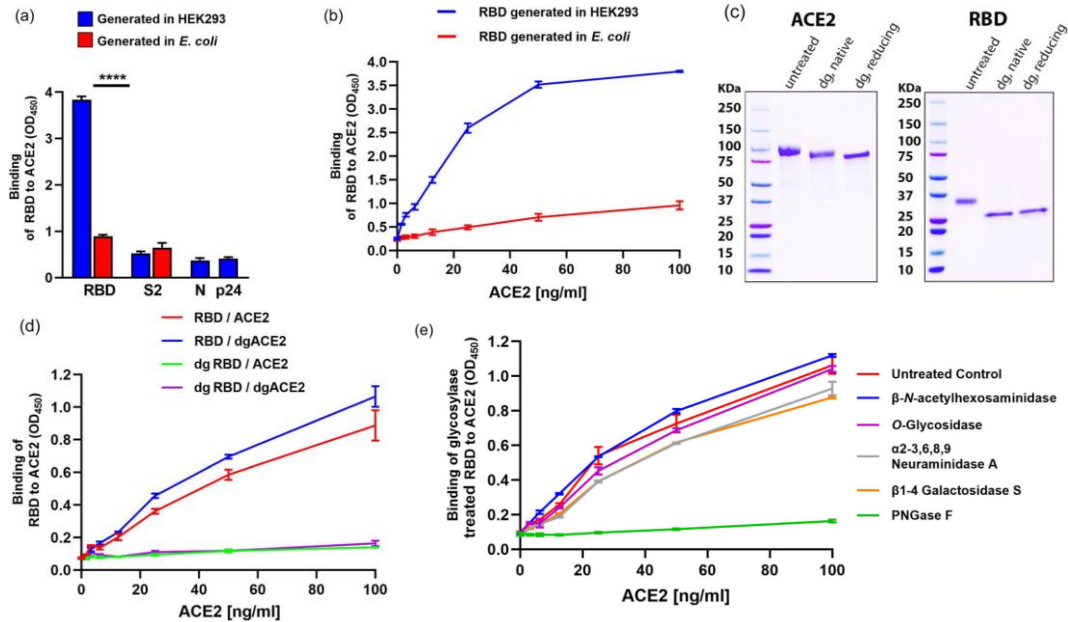


Figura 4: La glicosilazione N-linked di RBD è richiesta per il legame ad ACE2 – articolo in appendice.

La presenza del motivo Asn-Xaa-Ser/Thr, Xaa \neq Pro, ha suggerito che ci sono 22 siti putativi di glicosilazione sulla proteina spike. In particolare, i residui N331 e N343, localizzati su RBD, presentano una N-glicosilazione e, se vengono mutati in glutammina (Gln), l'infettività è ridotta. Per determinare se questi residui partecipano nell'interazione molecolare RBD/ACE2, Das et al hanno generato tre RBD ricombinanti mutanti: N331Q, N343Q e N331Q/N343Q (doppio mutante). L'attività di legame di questi mutanti è stata valutata usando un binding assay: il legame è abolito sia con il mutante singolo N343Q che con il doppio mutante N331Q/N343Q e significativamente diminuito con il mutante N331Q (figura 5a). Per misurare l'influenza dei siti N343 e N331 nell'infettività virale, sono stati creati pseudovirus caratterizzati dalla presenza della proteina spike di SARS-CoV-2 wild type o mutante (versioni mutate N331Q, N343Q, N331/N343Q) ed esperimenti la luciferasi^[6]. A seguito dell'ingresso del virus nella cellula ospite, la luciferasi viene espressa e quantificata attraverso la misura delle unità di luciferasi relative (RLU). L'espressione del wt e dei derivati mutati è stata confermata con un'analisi di immunoblot (figura 5b). Le cellule che sono state infettate sono cellule A549 e Vero, che hanno un'alta espressione di ACE2. I risultati hanno dimostrato che la doppia mutazione (N331Q/N343Q) comporta una sostanziale riduzione dell'infettività virale (inibizione > 80%); la mutazione singola N331Q, invece, causa una riduzione modesta (< 20%), mentre N343Q mostra una diminuzione del 50% (figura 5c,d). Sulla base di questi risultati, si dimostra che la mutazione di questi specifici siti di glicosilazione sulla proteina spike riduce significativamente l'infettività

pseudovirale. L'espressione della proteina ACE2 è stata testata in differenti linee cellulari (NIH-3T3, A431, A549, Vero) (figure 5e): le cellule A549 e Vero esprimono livelli significativi di ACE2, mentre le cellule NIH-3T3 e A431 no. La specificità del legame RBD/ACE2 è stata infine valutata con anticorpi: anticorpi anti-RBD (1H9; 4A9; 1F9), anti-S2 e anti-N (figura 5f).

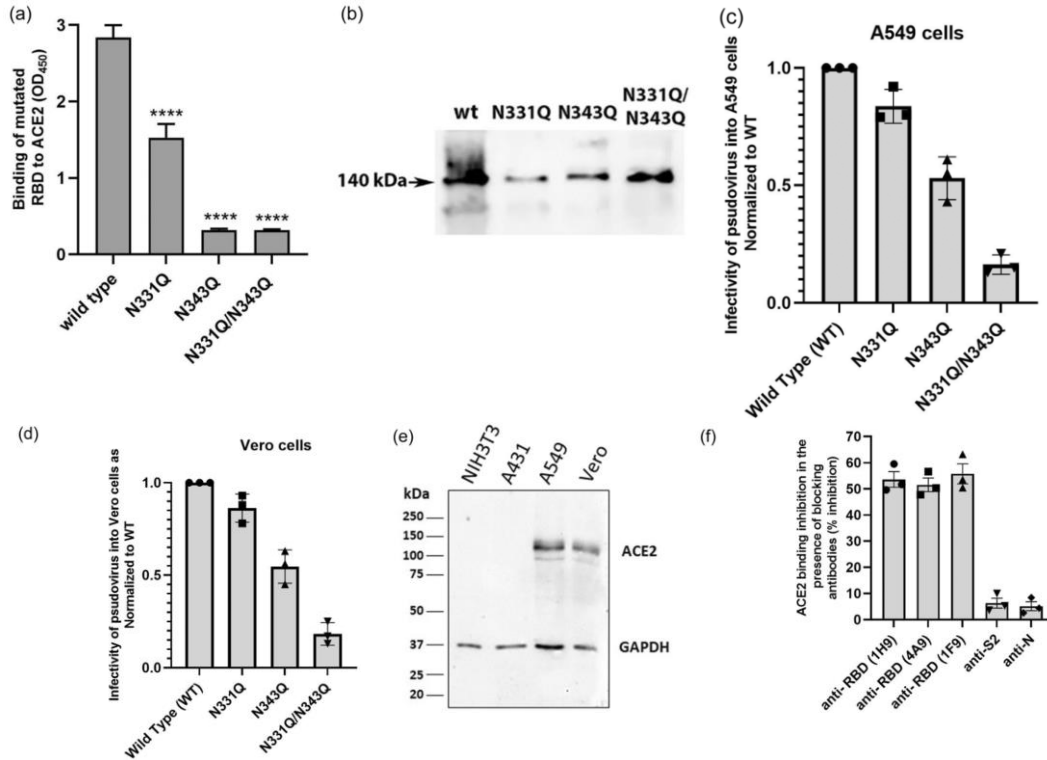


Figura 5: La glicosilazione di Asn433 di RBD è essenziale per l'interazione con ACE2 – articolo in appendice.

3.2 Identificazione degli epitopi glicani implicati nell'interazione spike-ACE2

Per esplorare le strutture dei glicani sul recettore umano ACE2 che interagiscono con la proteina S e per determinare la misura in cui queste interazioni dipendono da N331 e N343, un glycan array è stato ibridizzato con la proteina RBD wt o con i suoi derivati mutati. I dati del glycan array hanno mostrato che il legame di RBD con epitopi glicani contenenti un galattosio terminale era significativamente ridotto quando sia N331 che N343 erano mutati. Inoltre, è stata identificata la molecola glucidica di ACE2 che partecipa al binding: Gal-β-1,4-GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5. I ricercatori hanno osservato che le singole mutazioni (N331Q e N343Q) non riducevano il legame con tale epitopo, mentre il doppio mutante (N331Q/N343Q) rivelava una drammatica diminuzione nella capacità di binding (~ 80%) (figura 6a). Questo risultato è in linea con quelli ottenuti dal binding assay e dallo pseudovirus assay: le singole mutazioni bloccano il legame RBD/ACE2 e la penetrazione del virus in modo minore rispetto al doppio mutante. Basandosi su questi esiti, Das et al hanno ipotizzato un modello di interazione del doppio mutante con i glicani sulla superficie cellulare (figura 6b): il legame tra RBD e l'epitopo contenente un galattosio terminale presente sulla

superficie del recettore è diminuito quando i residui N331 e N343 sono mutati, a indicare che i glicani a livello di questi siti sono critici nell'interazione. Altri studi suggeriscono che i residui N90 e N322 su ACE2 possono interagire con RBD, in conseguenza del fatto che si trovano in sua prossimità^[7]. In uno studio precedente, Das et al hanno valutato la presenza della struttura Gal terminale sulla proteina ACE2 umana con un LC-MS/MS ad alta risoluzione e hanno trovato che è effettivamente presente in entrambi i residui (N90 e N322) (figura 6c); in particolare, la glicosilazione di N90 previene il legame con RBD, mentre quella di N322 lo favorisce. Infatti, strutture con un galattosio terminale sono maggiormente presenti su N322, ad indicare che l'assenza di acido sialico ha un ruolo nel legame tra RBD e ACE2.

Per ulteriore conferma dei risultati del glycan array, il recettore ACE2 è stato trattato con neuroamminidasi per rimuovere i residui di acido sialico e poi eseguito un binding assay. I ricercatori hanno osservato un aumento dose-dipendente nel binding: ACE2 trattato con neuroamminidasi lega con un'affinità maggiore (~25%) rispetto al wt (figura 6d). Tuttavia, in considerazione del fatto che esperimenti precedenti hanno dimostrato che la glicosilazione di ACE2 ha un impatto minimo sul legame con RBD, Das et al hanno ipotizzato che strutture glucidiche con un galattosio terminale possano essere presenti su tutta la superficie della cellula e avere capacità di aumentare il legame del virus con il recettore.

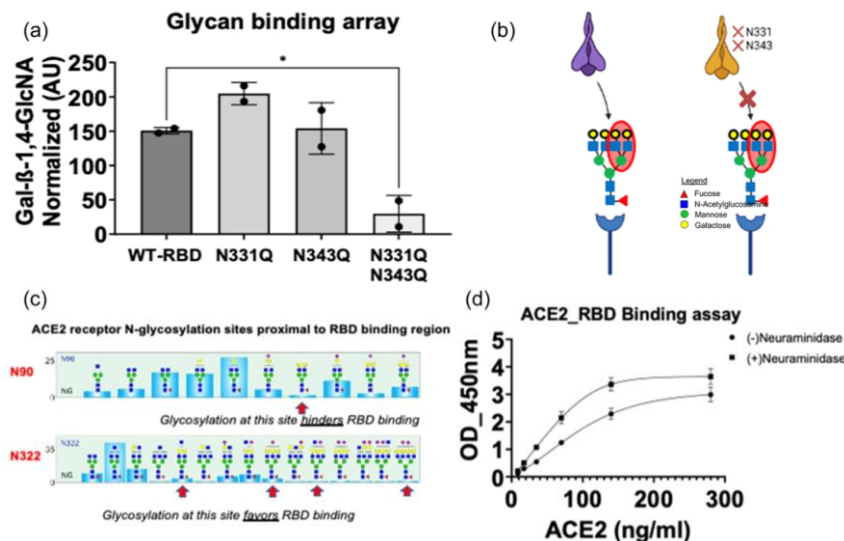


Figura 6: Identificazione dell'epitopo su ACE2 legato da RBD – articolo in appendice.

3.3 Ruolo della N-glicosilazione dei residui N331 e N343 nell'attivazione della risposta immunitaria pro-infiammatoria

Per comprendere la rilevanza patologica dalla proteina spike wt rispetto ai mutanti, i ricercatori hanno valutato i livelli di IL-6 nel plasma; questa citochina è infatti uno dei primi mediatori della risposta infiammatoria nei pazienti COVID-19 e la sua concentrazione varia a seconda della gravità dell'infezione. Dopo aver raccolto campioni di siero di pazienti, i livelli di IL-6 e di proteina spike sono stati quantificati con un sandwich ELISA. I risultati hanno mostrato una correlazione tra

i livelli di IL-6 e RBD ($R^2:0,802$; $p<0,0001$) (figura 7a). È stato quindi eseguito un antibody array a partire dai medium condizionati di cellule A549 infettate con virus wt e mutanti per identificare i cambiamenti più significativi nelle molecole di risposta al segnale dell'ospite rispetto al mock-infected control. È stato osservato che effettivamente ci sono importanti cambiamenti nei livelli di IL-6 (figura 7b). IL-6 viene down-regolato del 50% quando le cellule sono trasfettate con il plasmide doppio mutante di RBD, rispetto al wt. Per confermare tale risultato è stato effettuato un IL-6 ELISA (LOD 3 pg/mL) sul surnatante raccolto dalle cellule A549 wt e infettate; anche in questo caso si riscontra una down-regolazione di IL-6 in presenza del doppio mutante di RBD (figura 7c). Invece, in entrambi gli immunoassay, i mutanti singoli mostrano un cambio ambiguo nell'induzione di IL-6, suggerendo che l'effetto combinato delle due mutazioni è più robusto rispetto alle singole. Queste scoperte hanno suggerito che i due residui di RBD N331 e N343 contribuiscono almeno parzialmente all'espressione di IL-6 durante l'infezione da SARS-CoV-2.

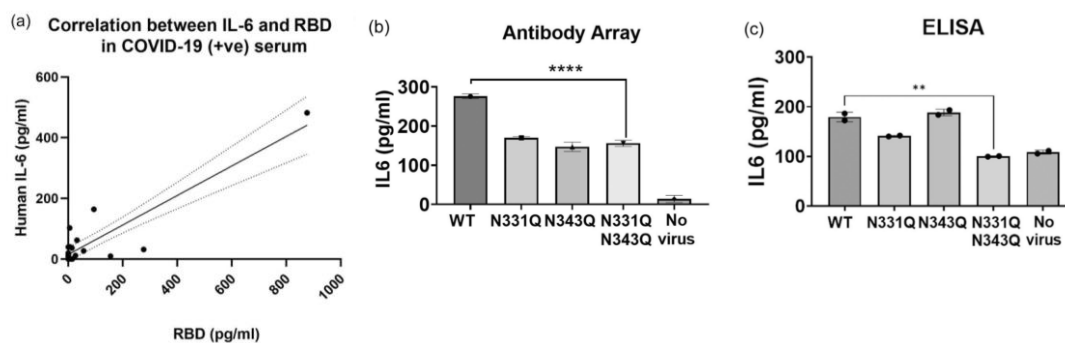


Figura 7: I mutanti di RBD non inducono l'espressione di IL-6 – articolo in appendice.

Recenti studi hanno stabilito che SARS-CoV-2 induce l'espressione di IL-6, così come la produzione di ROS e citotossicità in diversi tipi cellulari di mammifero. La trans-attivazione mediata da IL-6 del signaling pro-infiammatorio è operata attraverso una moltitudine di vie di segnale, che includono attivazione di Notch, NF- κ B e JAK-STAT. Das et al hanno cercato di valutare se l'esposizione delle cellule ospiti a RBD wt o mutanti influenzava l'espressione di IL-6. A tale scopo, hanno quantificato l'espressione del gene IL-6 umano in cellule epiteliali ed endoteliali (es: HEK-293, A549, HUVEC) con una real time-PCR. Tutte le linee cellulari sono state trattate sia con RBD wt che con le proteine mutanti per 24 o 48 ore a diverse concentrazioni. IL-6 ricombinante è stata usata come controllo positivo. I risultati hanno mostrato una minima up-regolazione dell'mRNA di IL-6 quando le cellule sono trattate con i mutanti singoli N331Q e N343Q, in confronto a cellule esposte a RBD wt o al controllo positivo. Invece, il livello di espressione dell'mRNA di IL-6 nelle cellule trattate con il doppio mutante N331Q/N343Q era simile al vehicle treatment (figura 8a-c). Questi dati supportano ulteriormente il coinvolgimento della glicosilazione N-terminale di N331 e N343 nell'attivazione della risposta immunitaria pro-infiammatoria (espressione IL-6). Per molte proteine virali, inclusa la proteina spike wt, è stata dimostrata la capacità di suscitare effetti

citotossici, tra cui apoptosi e distruzione delle giunzioni cellulari. La misurazione della citotossicità è stata fatta esaminando l'attività di LDH a partire da lisati di cellule trattate con diverse concentrazioni di proteine wt e mutanti. Il LDH assay ha rivelato che, in tutte e tre le linee cellulari (HEK-293, A549, HUVEC), la proteina RBD wt era citotossica, mentre i mutanti avevano una citotossicità non significativa, con il doppio mutante minore rispetto ai mutanti singoli. Questo trend nell'attività di LDH è consistente con i dati di espressione di IL-6.

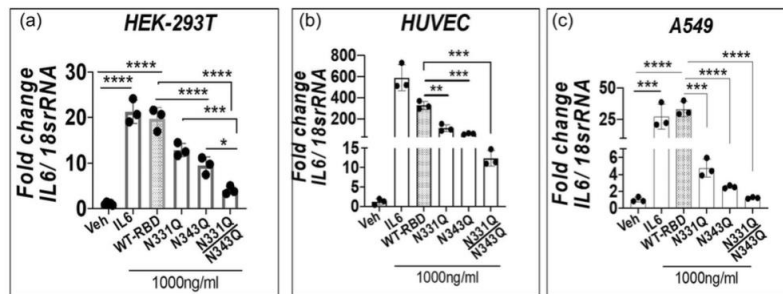


Figura 8: I mutanti N331Q e N343Q di RBD non inducono l'espressione di IL-6 – articolo in appendice.

4. Discussione

I risultati ottenuti dal binding assay dimostrano che ad essere cruciale nell'interazione S/ACE2 è la N-glicosilazione di RBD, in particolare a livello dei siti Asn331 e Asn343. Tali residui sono target della modifica post-traduzionale coinvolta nella patogenesi del virus. Questi dati confermano i risultati ottenuti da un altro gruppo di ricerca, che aveva dimostrato come la doppia mutazione N331Q/N343Q fosse in grado di ridurre in modo marcato l'infettività, mentre le mutazioni singole provocavano una diminuzione minore^[8]. La glicosilazione di N331 e N343 aumenta inoltre la stabilità della proteina spike, mantenendo la conformazione della subunità S1 nelle prime fasi del ciclo replicativo, e impedisce il legame di anticorpi, limitando anche il processo di presentazione antigenica. Mutanti di questi residui diminuiscono la capacità di penetrazione del virus, e quindi la sua fitness. I due glicositi rappresentano, quindi, una vulnerabilità che può essere sfruttata nella progettazione di vaccini che sfruttano i meccanismi dipendenti dalla glicosilazione utilizzati dalle cellule presentanti l'antigene per elaborare le glicoproteine virali^[9].

Das et al, inoltre, hanno identificato la molecola epitopo della proteina spike (Gal-β-1,4-GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5) e dimostrato che il legame tra tale molecola e il doppio mutante (N331Q/N343Q) di RBD è significativamente minore rispetto ai mutanti singoli N331Q e N343Q e alla proteina wt. I glicani aventi un galattosio terminale sono critici nell'interazione con RBD e sono presenti sui residui N90 e N322 di ACE2, che si trovano in prossimità della regione di legame a RBD. È stato osservato che la glicosilazione di N90 previene il legame di RBD, mentre quella di N322 lo favorisce, ed infatti epitopi con galattosio terminale sono relativamente più abbondanti su N322 rispetto ad altri glicani. È stata dimostrata anche l'importanza dell'assenza di un capping terminale

di acido sialico a livello di tali strutture glucidiche nel legame con RBD. I risultati infatti mostrano che, se ACE2 viene trattato con neuroamminidasi lega con affinità maggiore RBD, favorendo l'adsorbimento e l'ingresso del virus. In realtà il ruolo della glicosilazione di N90 nell'interazione con RBD è poco chiaro, anche se molti studi suggeriscono che la sua rimozione aumenti il binding tra RBD e ACE2^[10].

Per quanto concerne la patogenesi di SARS-CoV-2, una componente importante è la tempesta citochinica. In questo studio, Das et al hanno profilato l'espressione delle citochine e identificato che IL-6 è direttamente modulata da SARS-CoV-2 nel sistema dello pseudovirus. Le loro scoperte sono in linea con i recenti report nei quali è illustrato che l'infezione da SARS-CoV-2 o il trattamento di cellule epiteliali con la proteina spike induce il trans-signaling di IL-6 per attivazione dell'asse angiotensin II type 1 receptor (AT1), in modo da avviare una risposta iperinfiammatoria. L'infezione da SARS-CoV-2 induce, infatti, una cascata di segnale AT1-mediata e attiva fattori di regolazione trascrizionale come NF-kB e AP-1/c-Fos attraverso l'attivazione di MAPK. La cascata di segnale è in realtà indotta anche dal legame diretto di IL-6 circolante al suo recettore IL-6R. Il segnale indotto da IL-6 è stato dimostrato essere regolato dall'attivazione di Notch, NF-kB e JAK-STAT. È stato ipotizzato che mutazioni dei residui N331 e N343 di RBD potessero influenzare l'attivazione di IL-6, abolendo la tempesta citochinica mediata da tale molecola. I risultati della quantitative PCR hanno dimostrato, infatti, che sia i mutanti singoli che doppi della proteina RBD diminuiscono significativamente l'attivazione di IL-6 a livello trascrizionale in cellule epiteliali ed endoteliali umane. In particolare, il livello di IL-6 nelle cellule trattate con il doppio mutante era simile al controllo, suggerendo che entrambi i siti di glicosilazione contribuiscono all'induzione della risposta infiammatoria, i cui meccanismi restano non chiari. Inoltre, il doppio mutante non induce citotossicità (risultato LDH assay), concludendo quindi che la glicosilazione di RBD gioca un ruolo importante anche in tale processo e nell'infiammazione cellulare. Sebbene siano in fase di sviluppo diversi tipi di vaccini e terapie, una delle principali preoccupazioni è che le rapide mutazioni di SARS-CoV-2 possano limitare l'efficacia dei trattamenti vaccinali. Pertanto, identificare le basi molecolari della fase di ingresso virale, comprese modifiche post-traduzionali, potrebbe avere un importante risvolto terapeutico. Il modello di interazione RBD/ACE2 proposto nell'articolo suggerisce un target molecolare per il design di vaccini e cure che bloccano l'infettività virale e la tempesta citochinica.

Dall'articolo si conclude che la glicosilazione a livello dei siti Asn331 Asn343 è un aspetto chiave del meccanismo molecolare dell'interazione ospite-patogeno, dell'infettività e dell'attivazione dei pathway infiammatori down-stream nella cellula ospite.







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6. Appendice

N-glycosylation of the SARS-CoV-2 spike protein at Asn331 and Asn343 is involved in spike-ACE2 binding, virus entry, and regulation of IL-6

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic is an ongoing global public health crisis. The causative agent, the SARS-CoV-2 virus, enters host cells via molecular interactions between the viral spike protein and the host cell ACE2 surface protein. The SARS-CoV-2 spike protein is extensively decorated with up to 66 N-linked glycans. Glycosylation of viral proteins is known to function in immune evasion strategies but may also function in the molecular events of viral entry into host cells. Here, we show that N-glycosylation at Asn331 and Asn343 of SARS-CoV-2 spike protein is required for it to bind to ACE2 and for the entry of pseudovirus harboring the SARS-CoV-2 spike protein into cells. Interestingly, high-content glycan binding screening data have shown that N-glycosylation of Asn331 and Asn343 of the RBD is important for binding to the specific glycan molecule G4GN (Gal β -1,4 GlcNAc), which is critical for spike-RBD-ACE2 binding. Furthermore, IL-6 was identified through antibody array analysis of conditioned media of the corresponding pseudovirus assay. Mutation of N-glycosylation of Asn331 and Asn343 sites of the spike receptor-binding domain (RBD) significantly reduced the transcriptional upregulation of pro-inflammatory signaling molecule IL-6. In addition, IL-6 levels correlated with spike protein levels in COVID-19 patients' serum. These findings establish the importance of RBD glycosylation in SARS-CoV-2 pathogenesis, which can be exploited for the development of novel therapeutics for COVID-19.

KEYWORDS

ACE2, COVID-19, IL-6, N-glycosylation, receptor-binding domain, SARS-CoV-2, spike protein

Abbreviations: ACE2, angiotensin-converting enzyme 2; BHK21, baby hamster kidney 21; COVID-19, coronavirus disease 2019; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HEK293, human embryonic kidney 293; IL-6, interleukin-6; JAK-STAT, Janus kinase-signal transducers and activators of transcription; MERS-CoV, Middle Eastern respiratory syndrome; mRNA, messenger ribonucleic acid; NF- κ B, nuclear factor kappa b; qPCR, quantitative polymerase chain reaction; RBD, receptor binding domain; RLU, relative light units; RT, room temperature; RT-PCR, real-time polymerase chain reaction; S, spike; S1, spike subunit 1; S2, spike subunit 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TNF, tumor necrosis factor; VSV, vesicular stomatitis virus.

Tuhin Das, Shuhong Luo, and Hao Tang contributed equally to this study.

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic remains an urgent global public health concern, with over 676 million cases reported and over 6 million deaths worldwide as of May 2023.^{1,2} Despite worldwide vaccination efforts, the number of infections and fatalities will continue to rise for the foreseeable future. Numerous medications have been tested for the treatment of COVID-19, notably Remdesivir,³ but few therapies have demonstrated robust efficacy in clinical trials. Therefore, hospital care for COVID-19 patients will become commonplace worldwide, and treating complications such as cytokine storm and organ failure in severe cases will increase the burden on clinical care.

The causative agent of COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a member of the *Coronaviridae* family of viruses. Prior to 2002, *Coronaviridae* were known only as minor human pathogens, contributing to about 15%–25% of common colds.⁴ However, the emergence of an outbreak of severe acute respiratory syndrome (SARS) in 2002 caused by the coronavirus SARS-CoV propelled public health vigilance for *Coronaviridae*.⁵ To date, there are seven known coronaviruses of zoonotic origin that can cause human illness, with the coronaviruses MERS-CoV, SARS-CoV, and SARS-CoV-2 identified as being causal of SARS-like diseases.⁶

All coronaviruses are lipid-enveloped, positive single-strand RNA viruses. The lipid envelopes are made of three major components: two glycoproteins known as spike and membrane and a nonglycosylated envelope protein.⁷ The spike protein is a 1273 amino acid, a transmembrane protein that is essential for virus entry into host cells.⁸ Structurally, the spike is comprised of two subunits: an N-terminal S1 that contains a receptor-binding domain (RBD) and a C-terminal S2 domain that functions in host membrane fusion. The process of viral entry encompasses multiple stepwise interactions between host cell and viral proteins but is initiated by the attachment of SARS-CoV-2 RBD to the host cell surface protein angiotensin-converting enzyme 2 (ACE2). Thereafter, the S2 domain of the spike protein engages the type II transmembrane protease (TMPRSS2), which catalyzes the cleavage of spike in a process known as priming. Priming frees up the spike protein for fusion with the host cell membrane and subsequent entry into the host cell.⁹

A notable feature of the spike protein of SARS-CoV-2 is that it is extensively decorated with up to a hundred N-linked glycans, representing posttranslational modifications that are catalyzed by viral hijacking of the host's glycosylation pathways.^{10,11} Glycosylation of viral structures such as spike proteins contributes to the virus' host immune system evasion strategies by masking antigenic epitopes.¹⁰ Indeed, structural data, along with glyco-proteomic analyses, have proposed that extensive glycosylation of the spike protein shields against access by neutralizing antibodies.^{12,13} Importantly, glycans on the spike protein may also have a hitherto underappreciated role in host

cell receptor interactions and cell membrane fusion during viral entry into the cell. It is now well known that both the viral spike protein and human ACE2 receptor are extensively glycosylated, with a majority of the 22 N-glycosylation sites of spike and 7 N-glycosylation sites of ACE2-bearing carbohydrates.^{14–16} Specific distribution of glycan structures are important factors that are most likely to dictate the viral spike protein and host ACE2 receptor.^{17,18} However, the gap in the knowledge on the significance of glycosylation (N-terminal) of RBD in RBD-ACE2 binding and virus entry underscores an exigent need for characterizing the relative influence of residue-specific SARS-CoV-2 spike protein glycosylation in molecular recognition events comprising host–pathogen interaction.

Herein, we show that N-glycosylation of SARS-CoV-2 is essential for binding to ACE2 and for viral entry into cells. Specifically, our results reveal that the glycosylated residues Asn331 and Asn343 on RBD play key roles in binding to host cell receptor ACE2 and in viral infectivity. Furthermore, antibody array analysis revealed that IL-6 was significantly upregulated in conditioned media of cells infected with pseudovirus harboring the RBD protein. We also show that N-glycosylation of Asn331 and Asn343 is involved in the regulation of IL-6 expression and that IL-6 and RBD expression levels were correlated in COVID-19 patient sera. Finally, we report that the glycan (Gal- β -1,4-GlcNAc) may bind to N-glycans of Asn331 and Asn343. Collectively, these data show that N-glycosylation of Asn331 and Asn343 is crucial for the RBD/ACE2 interaction, for SARS-CoV-2 infectivity, and for the regulation of IL-6 expression. These data provide new insight into the molecular mechanism of SARS-CoV-2 pathogenesis and may inform the design and development of novel therapeutics against COVID-19.

RESULTS

N-linked glycosylation of RBD protein is required for binding to ACE2

Current research shows that glycosylation is an important component of RBD binding with human receptor ACE2 interaction for virus entry.^{17,19} However, the mechanistic pathway is not fully understood. Herein, to determine the role of glycosylation in the RBD-ACE2 interaction and to study in detail the role of glycosylation status during RBD-ACE2 binding, we developed a high throughput, in vitro screening method to measure molecular binding between the SARS-CoV-2 spike protein and the human ACE2 protein.²⁰ Microtiter plates were coated with either recombinant SARS-CoV-2 RBD, S2 domain, nucleocapsid (N) protein, or HIV p24 protein as negative control. Systematic incubation of these plates with recombinant human ACE2 confirmed that ACE2 was specifically bound to RBD and not any of the other viral proteins tested (Figure 1a). To test whether the binding affinity of RBD/ACE2 was impacted by mutations associated with the

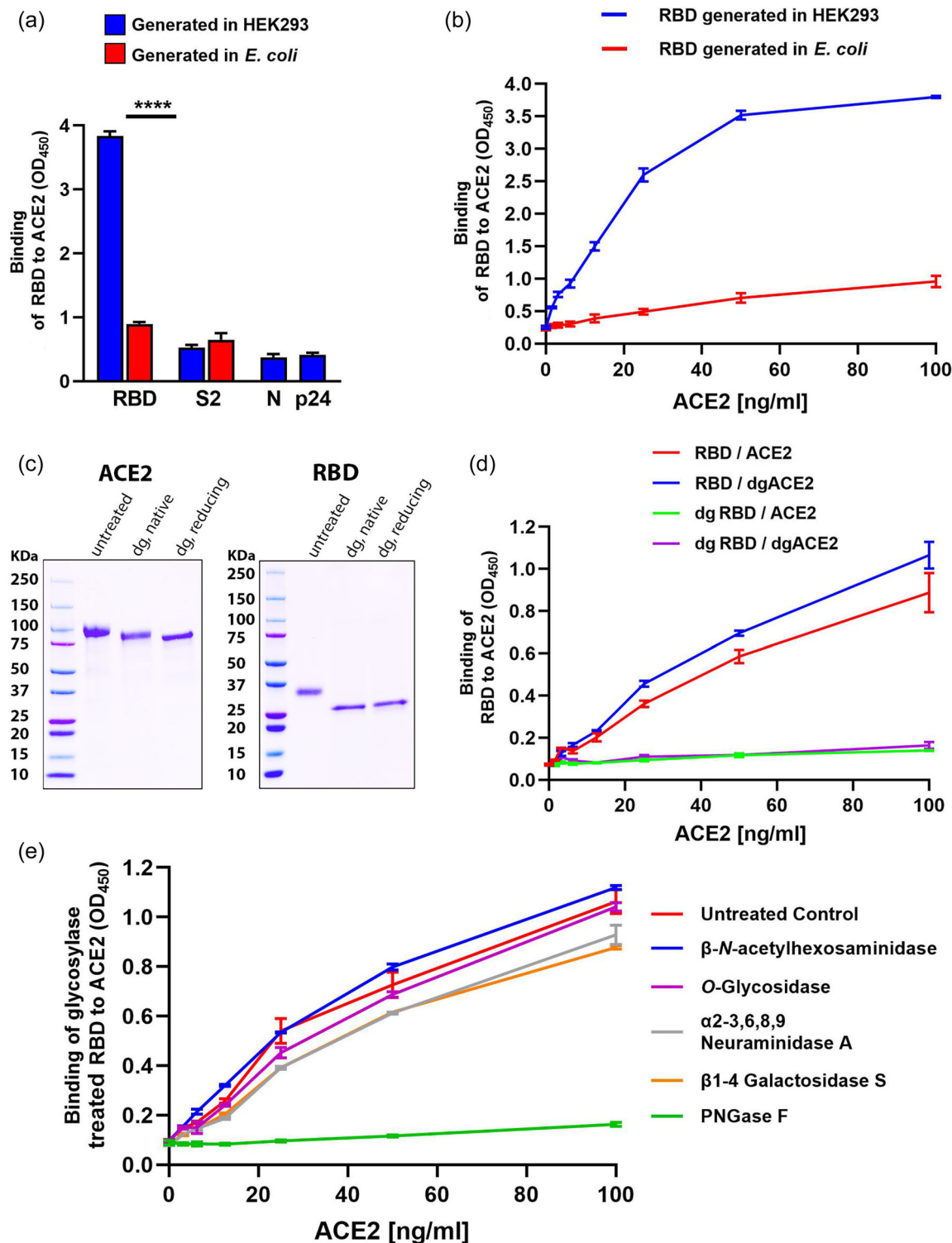


FIGURE 1 N-linked glycosylation of RBD protein is required for binding to ACE2. (a) Binding activity of SARS-CoV-2 RBD and S2 proteins derived from HEK293 cells or *Escherichia coli*. Binding was measured using an ACE2 binding assay, and nucleocapsid protein (N) and HIV p24 (P24) were used as negative controls. (b) Binding activity of RBD expressed either in HEK293 cells or in *E. coli* to titrated concentrations of ACE2. (c) Detection of purified and deglycosylated recombinant RBD and ACE2 proteins by SDS-PAGE. From left to right: untreated proteins, proteins deglycosylated (dg) under native conditions, proteins deglycosylated under reducing conditions. (d) Detection of RBD and ACE2 binding using the ACE2 binding assay under conditions where either or both RBD and ACE2 were deglycosylated (dg). (e) RBD was deglycosylated with individual enzymes: PNGase F, O-glycosidase, α 2-3,6,8,9 neuraminidase A, β 1-4 galactosidase S, or β -N-acetylhexosaminidase₆, prior to the ACE2 binding assay. Statistical significance was tested by *T* test for all experiments. **** $p < 0.0001$. $n \geq 3$ for all experiments.

omicron and delta variants of RBD, the *in vitro* binding assay was performed with recombinant omicron and delta variant RBD (Supporting Information S3: Figure 1), revealing a modest reduction in affinity only with the omicron variant. Moreover, recombinant SARS-CoV-2 RBD generated by over-expression within human HEK293 cultured cells bound to the ACE2 with significantly stronger affinity compared to recombinant RBD generated by expression within *Escherichia coli* (Figure 1a), in a dose-dependent manner (Figure 1b). These data indicate that eukaryotic-specific posttranslational modifications may influence ACE2/RBD binding affinity. Glycosylation, specifically N'-terminal glycosylation is a major eukaryotic post-translational protein modifying event that is hijacked by enveloped viruses.^{14,15} N'-terminal glycosylation of viral proteins evades the host immune response and aids in the viral life cycle.¹⁹

To determine the extent to which glycosylation influences ACE2/RBD binding affinity, RBD generated within HEK293 cultured cells was deglycosylated to remove both N and O-linked glycans, which resulted in a detectable shift in protein size (Figure 1c). We observed that untreated RBD bound to ACE2 in a dose-dependent manner, while deglycosylation of RBD abolished its binding to ACE2 (Figure 1d). Importantly, deglycosylation of ACE2 had a minimal impact on the binding interaction, suggesting that glycosylation of RBD, rather than of ACE2, is critical for the protein-protein interaction. To further interrogate the glycan linkages that function in ACE2/RBD binding, RBD was treated with multiple deglycosylation enzymes (PNGase F, O-glycosidase, α 2-3,6,8,9 neuraminidase A, β 1-4 galactosidase S, and β -N-acetylhexosaminidase_F) that target specific glycosylation links. Of the enzymes tested, only treatment with PNGase, which cleaves N-linked oligosaccharides, significantly lowered ACE2/RBD binding affinity (Figure 1e). These data suggest that N-linked glycosylation of RBD protein is required for binding to ACE2.

Glycosylation of RBD at Asn343 is essential for interaction with ACE2

The SARS-CoV-2 spike protein has 22 putative glycosites as determined by the presence of the Asn-Xaa-Ser/Thr, Xaa \neq Pro motif sequence.¹⁶ Of these sites, N331 and N343, are located on the RBD and have been shown to be N-glycosylated, and when mutated to Gln, were shown to reduce viral infectivity.^{16,17} To determine whether these residues participate in the RBD/ACE2 molecular interaction during binding, we generated three mutated recombinants of RBD, including N331Q, N343Q, and a N331Q/N343Q double mutation. The binding activity of these mutants to ACE2 was measured using the *in vitro* binding assay, which revealed that binding was abolished in both N343Q and N331Q/N343Q and was significantly lower in the N331Q mutant version of RBD (Figure 2a). To measure the influence of the N343 and N331 glycosites on infectivity,

we employed a pseudovirus system based on a method developed by Whitt et al.¹⁸ Briefly, we created a pseudovirus that expresses the wild-type SARS-CoV-2 spike protein, or mutants thereof, on a viral particle surface, while a plasmid encoding for luciferase is contained inside the viral particle. Upon viral entry into host cells, Luciferase (Luc) is expressed and quantified as a faithful readout of the extent of viral entry as a relative luciferase unit (RLU). In addition to wild type S, we generated pseudovirus expressing mutated versions, namely N331Q, N343Q, and a N331Q/N343Q double mutation on the viral surface. We confirmed the expression of viral wild-type and mutated derivatives using immunoblot analysis (Figure 2b). Measurement of luciferase activity within two different infected cell types with high ACE2 expression, namely A549 and Vero, revealed that double deletions at N331 and N343 resulted in a substantial reduction in viral infectivity (>80% inhibition). The single mutation N331Q caused a modest decrease of less than 20%, while N343Q exhibited a 50% decrease (Figure 2c,d). In accordance with the results of the *in vitro* binding assay, the data show that mutation of these specific glycosylation sites on the SARS-CoV-2 spike protein significantly reduces pseudoviral infectivity. ACE2 protein expression was tested in different cell lines, namely NIH-3T3, A431, A549, and Vero (Figure 2e). We confirmed that A549 and Vero cells expressed significant levels of ACE2, while NIH-3T3 and A431 cells did not. The specificity of RBD/ACE2 binding was then assayed with blocking antibodies, including anti-RBD (1H9; 4A9; 1F9), anti-S2, and anti-N antibody (Figure 2f).

Identification of glycan epitopes preferred by RBD protein

To explore the pertinent glycan structures on human receptors that interact with the S protein,^{21,22} and to determine the extent to which these interactions are dependent on N331 and N343, a glycan array was hybridized with the wild-type RBD protein or its mutated derivatives. The glycan array data showed that the binding of RBD with terminal galactose-bearing glycan epitopes was reduced significantly when both N331 and N343 were mutated. The candidate glycan molecule was identified as Gal- β -1,4-GlcNAc- β -1,3-(Gal- β -1,4-GlcNAc- β -1,6-)Gal- β -1,4-Glc-Sp5. Glycan array data analysis showed that each single mutation, either at N331 or N343Q, did not reduce the binding with Gal- β -1,4-GlcNAc- β -1,3-(Gal- β -1,4-GlcNAc- β -1,6-)Gal- β -1,4-Glc-Sp5; however, the double mutant (N331Q & N343Q), showed a dramatic reduction in binding (~80%) (Figure 3a). This is consistent with our results with the S-ACE2 binding assay and pseudovirus assay, which indicated that single mutations blocked RBD-ACE2 binding and viral entry to a lesser degree compared to the double mutant.

Based on our results, we hypothesized a model for the interaction of the double N-glycosylation mutant with

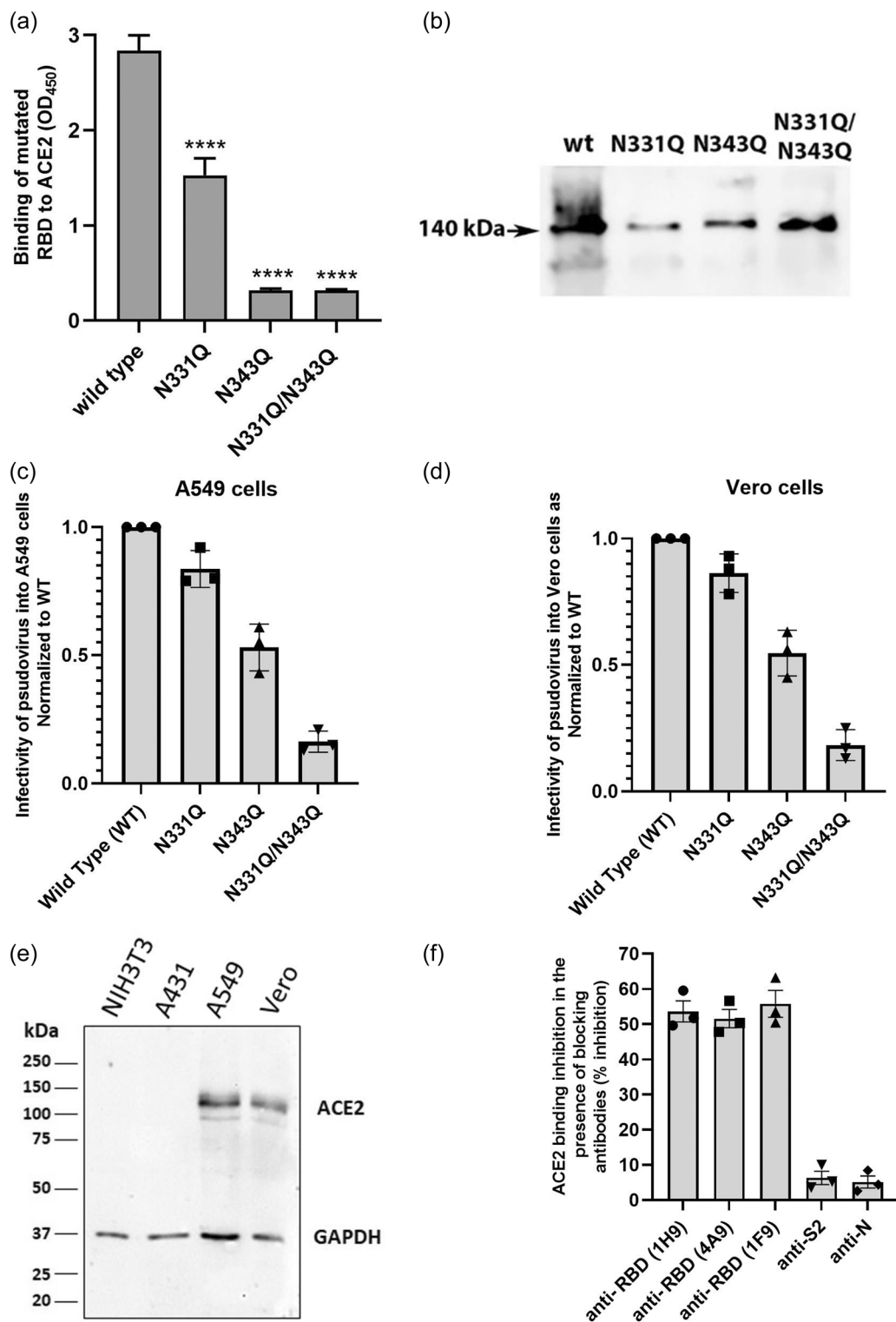


FIGURE 2 Glycosylation of RBD at Asn343 is essential for interaction with ACE2. (a) Detection of the binding of RBD wild type, or its N331Q, N343Q, and N331Q/N343Q mutated derivatives to ACE2 using the ACE2 binding assay. 40 ng/mL ACE2 was used in all assays. (b) Immunoblot analysis of recombinant SARS-CoV-2 RBD wild type or N331Q, N343Q, and N331Q/N343Q double mutated derivatives expressed within purified pseudovirus particles propagated in BHK21 cultured cells. (c) Detection of luciferase activity in A549 cultured cells infected with pseudovirus harboring S1 wild type, or its N331Q, N343Q, and N331Q/N343Q mutated derivatives. (d) Detection of luciferase activity in Vero cultured cells infected with pseudovirus harboring S1 wild type, or its N331Q, N343Q, and N331Q/N343Q mutated derivatives. (e) ACE2 expression levels (protein) in four cell lines, NIH3T3, A431, A549, and Vero, were detected by immunoblot. (f) The ACE2 binding was measured in the presence of blocking antibodies, including anti-RBD (1H9; 4A9; 1F9), anti-S2, and anti-N, to assess the specificity of ACE2 binding to RBD. Statistical significance was tested by *T* test for all experiments. *****p* < 0.001. *n* = 4/5 for all experiments.

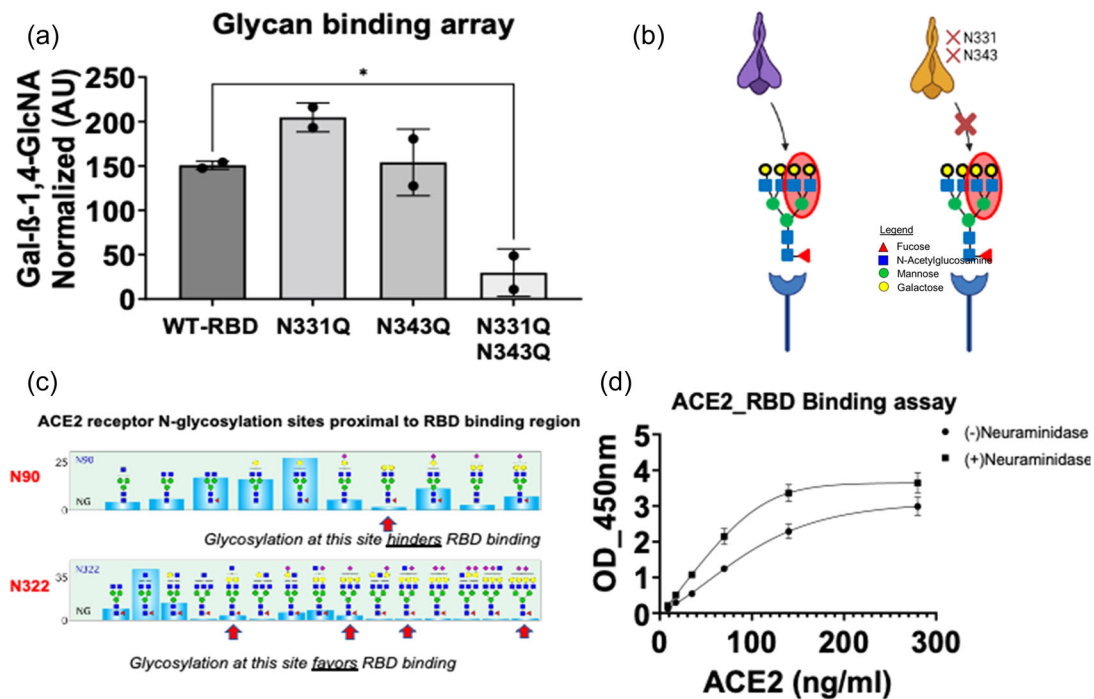


FIGURE 3 Identification of glycan binding epitope in the N-Glycan mutant site of RBD protein. (a) Identification of glycan recognition domains in the N331Q and N343Q mutants of RBD protein using a glycan array. Data represents two independent experiments. Data were analyzed using unpaired Tukey's test $*p < 0.02$ for statistical significance. (b) Schematic model of interaction inhibition in between double mutant (N331Q and N343Q) RBD mutant and WT RBD protein. Adapted from BioRender.com (2021). (c) N-Glycan distribution on sites N90 and N322 of human ACE2 receptor, which are proximal to the RBD binding region of S1 protein (red arrows show the glycans with terminal galactose). Modified from Shajahan and colleagues, with permission.²³ The glycan structure shown is drawn using the SNFG nomenclature, where the blue squares represent N-acetylglucosamine, the yellow circles represent galactose, the green circles represent mannose, and the red triangle represents fucose.^{23,24} (d) RBD-ACE2 binding increased in the presence of ACE2 treated with neuraminidase for 1 h at 37°C in a dose-dependent manner ($n = 2$ and duplicate for each set).

host cell surface glycans (Figure 3b). According to our model, the binding of RBD with the terminal galactose epitope is diminished when N331 and N343 glycan sites are mutated. This implies that glycans at these sites are critical for the interaction of RBD with terminal galactose structures on the cell surface receptor. Other studies suggest that residues N90 and N322 on ACE2 are predicted to interact with the RBD²² and are proximal to the RBD.²³ In a previous study, we evaluated the presence of this structure on human ACE2 by high-resolution LC-MS/MS and found that such terminal Gal structures are indeed present on both N90 and N322 of ACE2 (Figure 3c) and that N90 glycosylation on ACE2 prevents RBD binding while glycosylation at N322 favors RBD binding. Terminal galactose structures are displayed more at N322, which indicates that the terminal galactose structures without sialic acids may be critical in RBD binding with receptors (ACE2). We further confirmed our glycan array results by treating the receptor (ACE2) with neuraminidase to remove sialic acid residues, exposing the terminal galactose residue, and followed with the RBD-ACE2 binding assay. We observed a dose-dependent increase in binding. Neuraminidase-treated ACE2 binds with higher affinity (~25%) compared to wild-type RBD glycoprotein in the binding assay (Figure 3d).

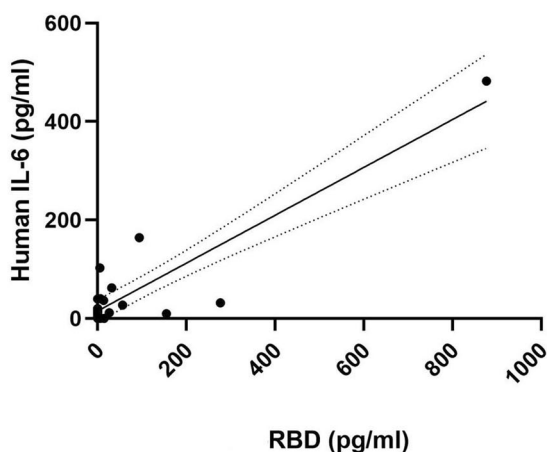
Since our earlier experiment showed that the glycans on ACE2 had a minimal impact on RBD binding (Figure 1d), we hypothesize that the terminal galactose glycan structures with which RBD interacts, may be displayed on the cell surface, and not necessarily on the ACE2 receptors. Cell surface glycans can enhance the viral binding with its receptors.²⁵ Thus, our observations indicate that glycans with terminal galactose expressed on the cell surfaces could facilitate binding of RBD, and such binding interactions are dependent on N-glycosylation at sites N331 and N343 of RBD.

Identification of IL-6 expression by antibody array analysis

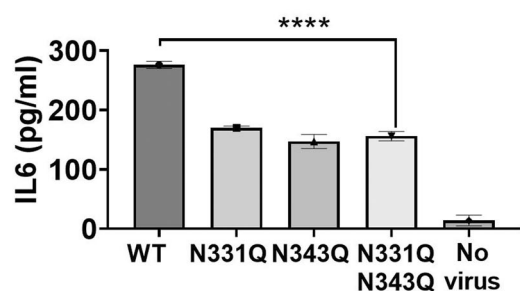
Next, we wanted to understand the pathological relevance of the wild-type (WT) spike-RBD in comparison with the N' terminal spike-RBD glycosylation mutants. Emerging lines of evidence suggest a strong correlation between plasma IL-6 levels and the severity of SARS-CoV-2 infection.²⁶⁻²⁸ Importantly, IL-6 has been suggested as one of the prime mediators of sustained proinflammatory response in COVID-19 patients. Therefore, we collected serum samples from subjects enrolled in the study and quantified IL-6 and spike protein levels by sandwich ELISA. Patients were

confirmed as COVID-19 positive or negative using an FDA-approved RT-PCR test. We also measured both IL-6 and RBD levels in COVID-19-infected patient's sera. Results from this study reveal a correlation between IL-6 and RBD levels ($R^2:0.802$; $p < 0.0001$) (Figure 4a). Next, to gain a better understanding of this finding, we performed an antibody array analysis of conditioned media from A549

(a) **Correlation between IL-6 and RBD in COVID-19 (+ve) serum**



(b) **Antibody Array**



(c) **ELISA**

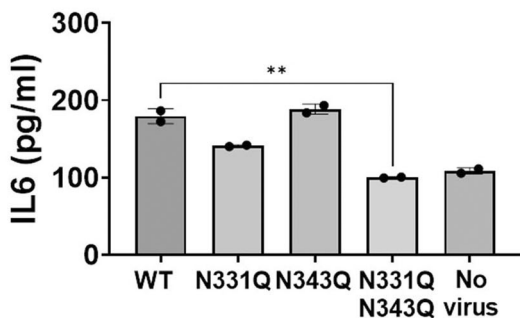


FIGURE 4 Glycosylation mutants of RBD do not induce IL-6 expression. (a) Correlation between IL-6 and RBD in COVID-19-positive sera. Serum samples from COVID-positive patients were analyzed by ELISA for IL-6 and RBD ($n = 21$). IL-6 was also measured in conditioned media from the pseudovirus assay (A549 cells) by (b) antibody array and (c) sandwich ELISA; all runs were performed in at least duplicate. Data were analyzed using one-way ANOVA ($****p < 0.0001$) and unpaired Tukey's test $**p < 0.0075$ for statistical significance.

cells infected with WT and mutant RBD viruses to identify the most robust changes in the host response signaling molecules compared to the mock-infected control. Our antibody array results revealed notable changes in IL-6 levels (Figure 4b). IL-6, a pro-inflammatory immune mediator, was downregulated by $>50\%$ when cells were transfected with double mutant RBD plasmid compared to WT RBD. To confirm this finding with a higher sensitivity assay, we performed an IL-6 ELISA (LOD 3 pg/mL) on supernatants collected from WT and RBD-infected A549 cells. We observed a similar downregulation of IL-6 in the presence of the double mutant RBD (Figure 4c). In both immunoassays, the single mutants exhibited an equivocal change in the induction of IL-6, suggesting that the combined effect of the two mutations is more robust than either alone. Taken together, these findings suggest that the two RBD residues N331 and N343 contribute at least partially to host IL-6 expression during SARS-CoV-2 infection.

Mutations at N331 and N343 sites of RBD exhibit blunted ability to elicit IL-6 expression

Recent studies have established that the SARS-CoV-2 S protein induces IL-6 mRNA levels as well as reactive oxygen species and cytotoxicity in various mammalian cell types.^{29–32} IL-6 transactivation of pro-inflammatory signaling is orchestrated via a multitude of signaling pathways, which include Notch activation, NF- κ B, and JAK-STAT activation.^{18,33–36} Hence, we investigated whether the exposure of host cells to wild-type RBD and N-glycan mutant RBD proteins influenced the expression of IL-6. We measured human IL-6 gene expression by RT-PCR in human epithelial and endothelial cells (i.e., HEK-293, A549, and HUVEC cell lines). All three cell lines were treated with either wild-type RBD or mutant RBD proteins for 24 or 48 h at varying concentrations in accordance with previous studies.³⁷ Recombinant IL-6 was used as a positive control protein for this study. Our results revealed minimal upregulation of IL-6 mRNA when cells were treated with either of the RBD single mutants (at N331Q and N343Q) when compared to wild-type RBD protein or recombinant IL-6 treatment. Interestingly, the expression level of IL-6 mRNA in cells treated with the N-glycan double mutant (N331Q & N343Q) was similar to vehicle treatments (Figure 5a–c). These results further support the involvement of the RBD in N-terminal glycosylations at N331 & N343 in the activation of pro-inflammatory immune response (i.e., IL-6 mRNA expression).

Several viral proteins, including wild-type spike protein, have been shown to elicit cytotoxic effects, including apoptosis and cell junction disruption in mammalian cells.^{38–41} Therefore, we next assessed the cytotoxic effects of the wild-type RBD in comparison with the N-terminal RBD glycosylation mutants. We measured cytotoxicity by measuring LDH activity from the lysates of cells treated

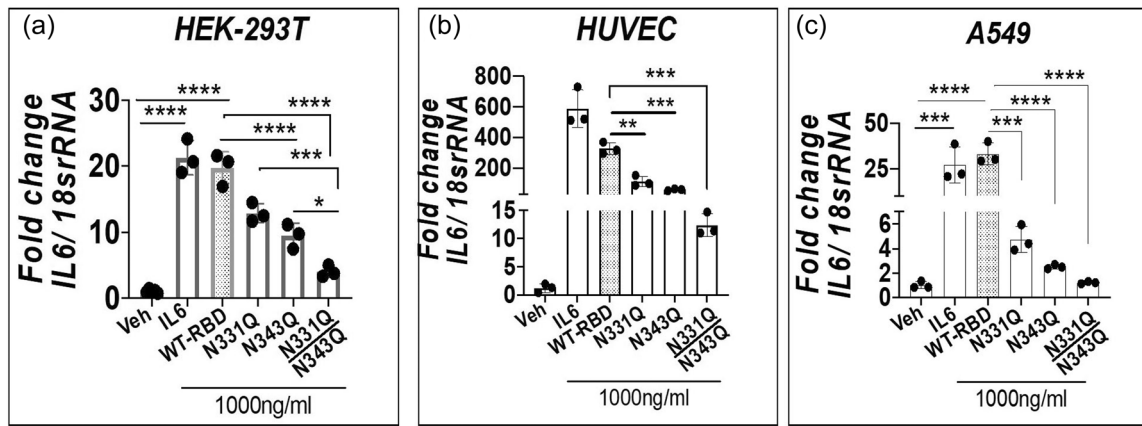


FIGURE 5 N-Glycan mutations at N331 & N343 of RBD protein did not induce IL-6 expression (a–c). Gene expression data for IL-6 mRNA was normalized with respect to 18sRNA (housekeeping control). Cell cytotoxicity was assessed in each cell line of the corresponding experiment in parallel using LDH measurements. LDH fold changes were normalized with corresponding protein amounts quantified in the cellular lysates using the BCA assay method (mg/mL). Each data represents the mean and SE from the $n = 3$ independent experiments performed in duplicate. Data were analyzed using one-way ANOVA with Tukey's test ** $p < 0.005$, *** $p < 0.001$, and **** $p < 0.0005$ for statistical significance.

with varying concentrations of WT and mutant proteins. The LDH assay revealed that, in all three cell lines, wild-type RBD protein was cytotoxic, whereas the RBD mutants had no significant cytotoxicity. (Supporting Information S3: Figure 2a–c). Moreover, the double mutant of RBD protein elicited still less cytotoxicity compared to either single mutant of RBD protein. These trends in LDH activity were consistent with that of the IL-6 expression data.

DISCUSSION

Glycosylation of SARS-CoV-2 spike protein has been widely recognized to have a significant role in COVID-19 pathogenesis.^{2,10,42,43} The RBD of spike protein is of particular interest because a substantial number of neutralizing antibodies with therapeutic benefits have been identified that directly bind to the RBD of the spike glycoprotein trimer.⁴⁴ Given the critical importance of spike protein in the viral fusion machinery, structural and functional aspects of putative glycosites on the RBD have gained considerable attention.

To study the role of glycosylation on SARS-CoV-2, we used an in vitro binding assay to show that glycosylation of the RBD protein, and not of ACE2, is critical for the S/ACE2 interaction. We further report that specific glycosylation sites on the SARS-CoV-2 spike protein, namely N331 and N343, are required for spike protein/ACE2 binding and viral infectivity. We, therefore, identify site-specific, N-linked, posttranslational modifications to the SARS-CoV-2 spike protein that are required for its pathogenesis. This finding is consistent with a previous report in which a double mutation at N331 and N343 of the SARS-CoV-2 spike protein markedly reduced infectivity, while single mutations at these residues exhibited less reduction in viral infectivity.¹⁷

Supporting the concept, prior research has reported on the function of specific-residue glycosylation in host cell and

pathogen interactions, innate immune responses, and inflammation.^{45,46} During viral pathogenesis, viruses co-opt the host's cellular glycosylation pathways to modify their own component proteins, which results in enhanced viral protein stability or can serve to occlude the binding of host antibodies to the virus. Furthermore, the decoration of viral proteins by glycans may hamper the host's ability to generate antibodies to epitopes containing the glycan by limiting antigen presentation by the HLA complex. While glycosylation of N331 and N343 of the SARS-CoV-2 spike protein may function in any of these processes, we show that mutating N331 and N343 lowers viral entry into host cells, which may point to these glycosites participating in maintaining the conformation of the S1 subunit during viral binding and entry.

Because glycosylation at N343 of the SARS-CoV-2 spike protein is essential to viral entry into the host cell, mutations of this residue, or defects in its glycosylation, would likely adversely affect virus fitness. Therefore, this glycosite represents a vulnerability that may be exploited in spike protein-based immunogen design for vaccine development. Indeed, neutralizing antibodies have been shown to directly contact RBD epitopes containing N343. It has been suggested that the identification of crucial glycosites such as those reported here would enable vaccine development strategies that leverage the glycosylation-dependent mechanisms used by antigen-presenting cells to process viral glycoproteins.³⁰

In this study, we identified the glycan epitope (Gal- β -1,4-GlcNAc- β -1,3-(Gal- β -1,4-GlcNAc- β -1,6-) Gal- β -1,4-Glc-Sp5) of spike protein, the binding of which is significantly reduced in the double mutant (N331Q and N343Q) compared to either the single (N331Q and N343Q) mutant or WT RBD protein. This implies that the glycans on the host receptors (i.e., human ACE2) with terminal galactose epitopes are critical in their interaction with the RBD. The N-glycosylations at N331 and N343 of spike protein might have roles in the interaction with

the receptors, and this interaction could be mediated through glycan structures with terminal galactose epitopes. Our findings are consistent with recently published molecular simulation studies that propose the critical roles of N-glycosylation in the RBD conformation changes and receptor binding.⁴⁷

The residues N90 and N322 on the ACE2 are proximal to the RBD binding region. Thus, we evaluated the presence of terminal galactose structures on human ACE2 and found that these structures are indeed present on both residues (depicted in Figure 3c).²³ N90 glycosylation of ACE2 prevents RBD binding while glycosylation at N322 favors RBD binding.^{23,48} Interestingly, the glycans with terminal galactose epitopes are relatively more abundant on N322 compared to other glycans. This suggests that these terminal galactose structures without sialic acid end capping on the ACE2 receptor may be critical in RBD binding. If this were true, we would expect ACE2 treated with neuraminidase to bind more tightly to RBD compared with neuraminidase untreated ACE2. Indeed, our results showed that RBD binds more strongly with neuraminidase-treated ACE2 in a dose-dependent manner (Figure 3d). We, therefore, speculate that this glycan interaction plays an important role in the S-ACE2 binding and potentially in viral docking and entry.

By contrast, the role of the N90 glycosylation in the interaction with the RBD is less clear. Interestingly, several studies suggest that removal of the glycosylation motif at N90 enhances the RBD binding with ACE2,^{49,50} while other studies have pointed to a limited role for the glycans on ACE2.⁵¹ Further study is required to fully understand the precise role of N90 glycosylation in RBD binding.

It is well known that cytokine storm, and particularly IL-6, are major components of the pathology of SARS-CoV2 infection; however, the mechanism for initiation of a hyper-inflammatory response and multiorgan damage from viral infection is poorly understood. Possible roles of IL-6 in COVID-19 pathogenesis have been discussed in several recent findings.^{52,53} Importantly, the functional significance of N-terminal glycosylation of RBD on cellular inflammation and cytotoxicity remains unclear. To address this knowledge gap, we first profiled cytokine expression and identified that IL-6, a pro-inflammatory signaling molecule, is directly modulated by SARS-CoV-2 in the pseudovirus model system. Our findings are in line with recent reports in which SARS-CoV-2 infection or spike protein treatment of epithelial cells significantly induced IL-6 trans-signaling by activation of the angiotensin II type 1 receptor (AT1) axis to initiate coordination of a hyper-inflammatory response.⁵⁴

It is evident that SARS-CoV-2 infection or spike protein induces AT1-mediated signaling cascade and thus activates transcriptional regulatory molecules such as NF- κ B and AP-1/c-Fos via MAPK activation, resulting in increased IL-6 release. IL-6 signaling cascade is also triggered by direct binding of circulating IL-6 protein to its cognate receptor, IL-6R.² IL-6-induced signaling has been shown to be regulated by Notch activation, NF- κ B, and JAK-STAT activation, which is an emerging area of investigation in the context of COVID-19 pathogenesis.^{18,33–36} Stimulation of

TLRs by bacterial or viral pathogen components or by pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α can activate cis-regulatory elements, leading to IL-6 synthesis. Recent studies have also shown that S protein treatment robustly induces IL-6 mRNA levels in mammalian cells.⁵⁴ Given the pathological relevance of the spike protein, we speculated that the mutations at N331 & N343 of RBD protein would affect IL-6 activation and thus abolish IL-6 mediated cytokine storm. Interestingly, our qPCR results (Figure 5a–c) showed that both single and double mutant RBD protein significantly reduced IL-6 activation at the transcriptional level in human epithelial and endothelial cells.

Although both single mutations at N331Q and N343Q RBD protein modestly reduced IL-6 expression, the level of IL-6 in cells treated with the double mutant (N331Q & N343Q) RBD protein was similar to that of control. This suggests that both glycosylation sites contribute to the RBD-induced inflammatory response (i.e., IL-6 production), whose mechanisms remain unclear in our present investigation. This observation was consistent with the finding that IL-6 should be very minimal either in normal sera or in COVID-19-recovered patients.⁵⁵ In addition, our findings revealed that the double mutant did not induce cytotoxicity as observed in the LDH assay experiments. Given the consistency in our findings from multiple independent mammalian cell lines, it can be thus concluded that the glycosylation of RBD plays an important role in RBD induced cytotoxicity and cellular inflammation. Our IL-6 qPCR results were consistent with the binding assay and cell-based pseudovirus assay data (Figures 1 and 2). Furthermore, the dependence of the viral cell entry on the integrity of the RBD/ACE2 interaction suggests that a vaccine targeting this epitope may retain efficacy through seasonal antigenic drift.² The spread of COVID-19 is continuing worldwide with little or no sign of slowing down. Although multiple types of vaccines and therapeutics (including antibodies and small molecules) are being developed, a major concern is that fast mutations of SARS-CoV-2 may restrict the efficacy of vaccines or treatments.³² Therefore, identifying the molecular underpinnings of viral entry into the host cells, including posttranslational modifications, is of crucial value for the development of new therapies to treat COVID-19. We contribute to these endeavors by showing that glycosylation modifications, likely by molecule (Gal- β -1,4-GlcNAc- β -1,3-(Gal- β -1,4-GlcNAc- β -1,6-) Gal- β -1,4-Glc-Sp5) moieties at specific residues to the SARS-CoV-2 spike protein, is required for viral binding and entry. Because there is considerable existing evidence that human neutralizing antibodies target the RBD of spike protein, and some of which have shown therapeutic promise,^{29,31,35,36} characterization of glycosylation modifications at N331 and N343 is critical knowledge to inform the development of neutralizing antibodies against COVID-19.

In this study, we show the glycosylation of N331 and N343 residues of RBD protein to be critical for the induction of pro-inflammatory signaling (i.e., IL-6 expression) and

cytotoxicity (LDH release). The glycosylation of these 2 residues is, therefore, a key aspect of the molecular mechanism of the host–pathogen interaction as well as its downstream inflammatory pathways in the host cell. Our proposed model (Figure 6) provides a molecular target for the design of vaccines and therapies to block viral infectivity and cytokine storms.

MATERIALS AND METHODS

Spike/ACE2 binding assays

We employed an ELISA methodology described previously²⁰ to analyze the interaction of the RBD with ACE2. In brief, 96-well microplates were coated with

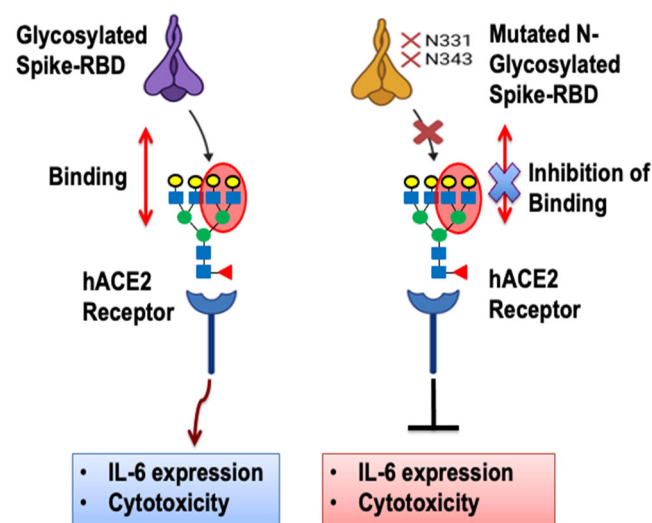


FIGURE 6 Proposed model: Mutations in N-glycosylation sites at N331 and N343 residues of RBD protein inhibit S-ACE2 binding, IL-6 expression and cytotoxicity. I confirm and agree with the possibility that my figure images might be selected and used as a journal cover image at issue publication.

recombinant RBD. Recombinant human ACE2 protein was added in varying concentrations, followed by washing, before incubation with goat anti-ACE2 antibody. After the addition of HRP-conjugated anti-goat IgG, signals were generated with 3,3',5,5'-tetramethylbenzidine substrate and plates read by colorimetry at 450 nm. Recombinant proteins used for the binding assay are listed in Table 1. Deglycosylation of the proteins was performed under native or reducing conditions using Protein Deglycosylation Mix II (New England Biolabs, cat. P6044) or the individual deglycosylases, PNGase F, O-glycosidase, α 2-3,6,8,9 neuraminidase A, β 1-4 galactosidase S, and β -N-acetylhexosaminidase_f (New England Biolabs) following the manufacturer's instructions. SDS-PAGE electrophoresis and immunoblot were performed using standard protocols. Mouse anti-SARS-CoV-2 S1 protein antibody (RayBiotech, cat. 130-10864) was used at 1 μ g/mL.

Cell culture and treatments

Two cell lines with high ACE2 expression (Vero and A549) and were purchased from the ATCC. Vero C1008 is an African monkey kidney cell line (ATCC® CRL-586™). A549 is an adenocarcinomatoid human alveolar basal epithelial cell line (ATCC® CCL-185™). BHK21/WI-2 is a baby hamster kidney cell line (Kerafast catalog # EH1011). BHK21 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5% FBS (Atlanta Biologicals) and penicillin-streptomycin (Corning) at 37°C in 5% CO₂. Vero and A549 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1x penicillin-streptomycin at 37°C with 5% CO₂. Δ G-luciferase (G* Δ G-luciferase) VSV pseudotypes carrying SARS-CoV-2 spike or VSV-G-kan were produced from BHK21 cells. The pseudoviral particle titration and neutralization assays were conducted using Vero and A549 cells.^{18,34}

TABLE 1 Recombinant proteins used in binding assays.

Protein	Species	GenBank	Expressed region	Expression system	Source
S1 subunit, RBD	SARS-CoV-2	QHD43416	Arg319-Phe541	HEK293	RayBiotech (cat. 230-30162)
S1 subunit, RBD	SARS-CoV-2	QHD43416	Arg319-Phe541	<i>Escherichia coli</i>	RayBiotech (cat. 230-01102)
S2 subunit	SARS-CoV-2	QHD43416	Met697-Pro1213	HEK293	RayBiotech (cat. 230-30163)
Nucleocapsid	SARS-CoV-2	QHD43423	Met1-Ala419	HEK293	RayBiotech (cat. 230-30164)
ACE2	Human	Q9BYF1	Gln18-Ser740	HEK293	RayBiotech (cat. 230-30165)
p24	HIV	Q9WMW5	Ser5-Cys207	<i>E. coli</i>	RayBiotech (cat. 230-00505)
S1 subunit, RBD, L452R/T478K mutant	SARS-CoV-2	QHD43416	Arg319-Phe541	HEK293	RayBiotech (cat. 230-30208)
Spike RBD, B.1.1.529	SARS-CoV-2	YP_009724390.1	Arg319-Phe552	HEK293	SinoBiological (cat. 40592-V08H121)

Pseudovirus plasmids

pCAGGS-G-kan plasmid was purchased from Kerafast (EH1025-PM). The full-length cDNA fragment encoding SARS-CoV-2 (Wuhan-Hu-1 strain) wild-type spike glycoprotein (GenBank accession # QHD43416, Met1-Thr1273) was cloned into pCAGGS-G-kan vector named wt-S/pCAGGS vector. Three S mutant constructs (N331Q, N343Q, and N331/343Q/pCAGGS) were generated by DNA mutagenesis using the mother vector wt/pCAGGS as the template. All recombinant constructs were confirmed by DNA sequencing. Endotoxin-free cell transfection-grade plasmid DNA was extracted using a MaxiPrep plasmid preparation kit (Omega Bio-Tek, Norcross, GA, cat. D6922) according to the manufacturer's protocol.

Pseudovirus production

G*ΔG-luciferase VSV (purchased from Kerafast: EH1020-PM) and VSV-SARS-CoV-2 pseudotyped viral particles were generated as previously described.^{18,34} One day before transfection, BHK21 cells were cultured in a 100 mm cell culture dish at the concentration of 3×10^6 cells and incubated overnight at 37°C in 5% CO₂ to reach 70%–90% confluence. Using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions, BHK21 cells were transfected with 16 μg pCAGGS-G-kan plasmid. The transfected cells were subsequently infected with G*ΔG-luciferase VSV at a MOI of 0.1. These cells were incubated at 37°C in 5% CO₂ for 1.5 h, followed by the addition of fresh complete DMEM. At 24 h postinfection, culture supernatants containing G*ΔG-luciferase VSV were harvested, and aliquots were stored at –80°C until use. To generate SARS-CoV-2 pseudovirus, BHK21 cells were transfected with 16 μg pCAGGS-SARS-COV-2 spike plasmid. The transfected cells were subsequently infected with G*ΔG-luciferase VSV at a MOI of 3–5.

Pseudovirus titration

The pseudovirus titers were determined in Vero cells, A549 cells, and BHK21 cells. Cells were seeded in 96-well white cell culture plates with a clear bottom (Corning, 3610) and were infected with 100 μL of pseudovirus 2-fold serially diluted in serum-free DMEM (the initial dilution was 10-fold). The first row was treated as the control without pseudovirus (mock infection). After 1.5 h of incubation at 37°C in 5% CO₂, 100 μL fresh DMEM containing 10% FBS and penicillin-streptomycin were added. At 24 h postinfection, the relative luminescence units (RLU) of luciferase activity were determined using the ONE-Glo EX Luciferase Assay System (E8110; Promega, Inc.) and a luminometer (BioTek Synergy HT) according to the manufacturer's instruction.³³

Glycan array

A glycan array chip with a panel of 300 glycans (cat# GA-Glycan-300; RayBiotech Inc.) was used as a high throughput screen to determine interactions between RBD protein (WT vs. N331Q vs. N343Q and N331Q/N343Q) and the arrayed carbohydrates. In brief, glycan array slides were brought to RT and blocked with diluent for 30 min. Four hundred microliters of RBD and mutant proteins (50 ng/mL) were incubated in the well for 2–3 h, followed by washing. Biotinylated primary antibody (cat# 130-10877; RayBiotech, Inc.) at 20 ng/mL was added and incubated for 1 h, followed by washing. Then, mouse secondary at dilution 1:5000, which can detect IgG, was added and incubated for 1 h, followed by washing. After washing, Cy3 equivalent dye-conjugated streptavidin at RT for 1 h with gentle rocking. After washing, the glass slide was further washed with 30 mL of de-ionized or distilled water for 5 min. The signals were visualized through Axon GenePix, a laser scanner, and followed by image analysis and quantification using microarray analysis software (GenePix 4000).

Neuraminidase treatment of ACE2 and S-ACE2 binding assay

Neuraminidase treated ACE2 was incubated for 1 h at 37°C, including control (without enzyme). RBD-ACE2 binding assay was used to see the binding efficiency between RBD and ACE2 (with and without neuraminidase treated ACE2) in a dose-dependent manner. At the end of the experiment, we calculated the binding change (%) between RBD & ACE2 under neuraminidase treatment.

Antibody array processing and data analysis

The L-1000 Array (cat# AAH-BLG-1000; RayBiotech, Inc.) is a label-based semi-quantitative array for the detection of 1000 human proteins, where each protein-specific antibody is fixed on the glass surface while samples are biotinylated. Cell culture media samples were processed according to the manufacturer's protocol. Briefly, 120 uL cell culture media were first purified with a desalting spin column, then biotinylated at ambient temperature for 30 min. The reaction mixtures were then passed through another desalting spin column to remove the free biotin. A 10x sample dilution was processed on the arrays, and the slides were scanned using the Innopsys 710AL scanner. The data were extracted with Mapix software and further analyzed with array-specific Excel software. The Kiloplex Antibody Array (cat# QAH-CAA-X00; RayBiotech, Inc.) is a sandwich-based quantitative array for detecting 1000 human proteins. The arrays were processed with a twofold sample dilution following the manufacturer's protocol. After processing, the slides were scanned in XDR mode using the Innopsys 710AL scanner, and the data were extracted by

using Mapix software. Array-specific data analysis software was used for data computation.

The RayBio® Human IL-6 ELISA kit (cat# ELH-IL6) is an *in vitro* ELISA for the quantitative measurement of human IL-6 and was used to determine the IL-6 level in conditioned media from the pseudovirus assay in parallel with the same experiment.

Real-time PCR analysis

For measuring IL-6 mRNA expression, total RNA was isolated from WT-RBD, Mutant proteins, and human recombinant IL-6 treated HEK-293T and HUVEC cells, respectively, using RNeasy Mini kit (QIAGEN), and cDNA synthesis was conducted using iScript cDNA synthesis kit (Bio-Rad). qPCR assay was performed by subjecting 500 ng of cDNA using power TRACK Sybr mix (Thermo Fisher Scientific) using primers specific for human IL-6 (forward: 5'-TCGGTCCAGTTGCCTTCT-3' and reverse: 5'-TGAGATGCCGTCGAGGAT-3') and 18srRNA (forward: 5'-TTGGTGGAGCGATTTGTCTG-3' and reverse: 5'-ATCTCGGGTGGCTGAACG-3') as per manufacturer's instructions. The expression levels of IL-6 mRNA were normalized to that of 18srRNA levels. Relative expression of IL-6 mRNA in untreated control and treated samples is expressed as $2^{-\Delta C_t}$ values as described previously, and fold changes are calculated by comparing the $2^{-\Delta C_t}$ values of the treated sample with that of untreated control.

Lactate dehydrogenase (LDH) assay

To determine cytotoxicity, LDH release was measured with the LDH Cytotoxicity Assay Kit (Pierce) according to the manufacturer's protocol. HEK-293, A549, and HUVEC cells were plated, respectively, into 12-well plates and maintained overnight before treatment, following which the cells were treated with WT-RBD, Mutant proteins, and human recombinant IL-6 at 1000 ng/mL for 24 h. After treatment, the cell supernatants were collected by centrifugation. Supernatants were assayed for LDH in triplicates as per the manufacturer's protocol. Absorbances were measured at 490 and 680 nm using a spectrophotometer (Biotek). Absorbance values were normalized to the protein amounts in the analyzed lysates by BCA quantification (Pierce; Thermo Fisher). Fold change in cytotoxicity was calculated based on the difference compared with the LDH-positive control provided with the kit.

Patient's serum collection and IL-6 and RBD quantification

This is a single-center prospective cohort study performed at PanoHealth LLC. Blood samples were collected from adult

patients and were selected based on COVID-19 diagnosis by FDA-approved RT-PCR test. Blood samples were processed according to Sterling IRB: 8291-BZhang. Serum was subsequently used for IL-6 and RBD measurement.

Whole blood was collected in tubes containing separation gel (VACUETTE). After centrifugation, the serum was collected and stored at -80°C . IL-6 (Cat# ELH-IL6; RayBiotech, Inc.) and RBD (Cat# IEQ-CoVS1RBD-IgG-1; RayBiotech, Inc.) were quantified using an ELISA kit, according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean \pm SEM obtained from an appropriate number of independent experiments conducted in either at least duplicate or triplicates, as stated. Statistical significance was assessed using GraphPad Prism software, version 8.2.1 (GraphPad Prism Software). Unpaired or paired two-tailed *t* tests were used, as stated, to analyze data involving a direct comparison of an experimental group with a control group. One- or two-way ANOVA was used with repeated measures for appropriate experimental groups as stated, followed by Tukey's and Bonferroni's multiple comparison correction. For *in vitro* quantitative-PCR and LDH release experiments, we performed one-way ANOVA to determine the significance of target gene expression in control versus treatment groups. The reported *p* values were adjusted to account for multiple comparisons. For all statistical tests, a two-sided confidence level of $p < 0.05$ (95% CI) was accepted for statistical significance.

AUTHOR CONTRIBUTIONS

Ruo-Pan Huang, Tuhin Das, Shuhong Luo, and Hao Tang conceived and designed the experiments. Tuhin Das, Shuhong Luo, Hao Tang, Jianmin Fang, Sabyasachi Dash, Steven Huang, Shehuo Xie, Gordon F. Huang, Blake Anderson, and Benyue Zhang performed all experiments. Tuhin Das, Shuhong Luo, Hao Tang, Sabyasachi Dash, and Ruo-Pan Huang analyzed all data. Asif Shajahan, Lauren Pepi, Parastoo Azadi, and Jinqiao Lu contributed intellectually and assisted in figure design. Tuhin Das, Valerie S. Jones, and Ruo-Pan Huang wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

Shuhong Luo, Hao Tang, Jinamin Fang, Yingqing Mao, Haw-Han Yen, Steven Huang, Valerie Sloane Jones, Shehuo Xie, Gordon Huang, Jinqiao Lu, Blake Anderson, Benyue Zhang, and Ruo-Pan Huang are employees of and have a financial stake in RayBiotech, Inc.












DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by IRB (ID# 8291-BZhang). The patients/participants provided their written informed consent to participate in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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